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**DEVELOPMENT OF A GREEN
TECHNOLOGY-BASED NUTRACEUTICAL
FORMULATION USING SUPERCRITICAL
CO₂**

MICROFLUIDIC-ASSISTED FORMULATION AND CHARACTERIZATION OF
QUERCETIN LIPOSOMES AND VS10 LIPOSOMES.

SSD: Molecular Biology [BIO/11]

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Abstract

The thesis is divided into three chapters, where the *Chapter 1 - Development of a Green Technology-Based Nutraceutical Formulation using Supercritical CO₂* focuses on developing nanocrystals as a delivery system to preserve the bioavailability of bioactive components and thereby enhancing the parameters such as solubility, permeability, and stability. Supercritical carbon dioxide (Sc-CO₂) technology offers a green, sustainable method for both extracting bioactive substances and producing delivery systems. Quercetin as a bioactive component was chosen based on its multifaceted functions but because of its hydrophobicity, low bioavailability nanocrystals an encapsulated system were prepared. Quercetin nanocrystals, produced using the Solvent Antisolvent (SAS) method, was encapsulated using Pluronic F127 and 2-Hydroxypropyl β -cyclodextrin (2-HP- β -CD) improved solubility and stability, as confirmed by FTIR, DSC, XRD analysis. Thermal and kinetic studies demonstrated stability up to 100°C and sustained drug release in intestinal and gastric media. Quercetin nanocrystals were prepared using the Supercritical CO₂ and Liquid CO₂, as an antisolvent. The nanocrystals prepared using the CO₂ based mechanism were compared with the batch method of preparation namely Solvent injection method. The results obtained showed comparatively better solubility, stability, molecular interactions and release profile of the nanocrystals prepared using the CO₂ based technology.

Further, *Chapter 2 - Microfluidic-Assisted Formulation and Characterization of Quercetin-Loaded Liposomes for Enhanced Oral Delivery* was the PhD project performed in collaboration with Galenica Senese. The aim was to obtain an optimized liposomal delivery system as an antioxidant supplement of quercetin using food-grade, FDA-approved excipients. The liposomal preparation was achieved using the microfluidics technology. These formulations demonstrated consistent particle sizes (100–150 nm), low polydispersity index (PDI < 0.3), and high stability, as validated by TEM analysis. Safety studies confirmed no cytotoxic effects on healthy cells at concentrations above 100 μ M.

In *Chapter 3 - Microfluidic-Based Development of VS10-Loaded Liposomes as a Chemotherapeutic for Targeted Pin1 Inhibition* involved the microfluidics technology that enabled the development of VS10 liposome. VS10 is a PIN1 inhibitor which interacts with the critical active sites and mediate the degradation of Pin 1 but VS10 is a BCS class 2 drug and thus provide low solubility and bioavailability. Hence it was encapsulated in liposomes that achieved 80% efficiency, significantly reducing IC₅₀ values in ovarian cancer cell lines.

This multidisciplinary approach highlights the potential of green technologies and advanced delivery systems in maximizing the therapeutic and preventive benefits of bioactive compounds.

Chapter 1

*Development of a Green Technology-Based
Nutraceutical Formulation using Supercritical CO₂*

1. Introduction

1.1 Nutraceutical World

Nutraceuticals will be considered components of traditional and exotic foods that have the potential to augment human health. The substance may (a) be part of an intact food source, such as the polyphenols naturally occurring in blueberries; or (b) be part of a processed food, such as polyphenols from blueberries incorporated into a smoothie; or (c) be a fortified or enriched substance in a food, such as polyphenols added to a functional yogurt; or (d) be provided in supplemental form, such as polyphenol-rich capsules, tablets, liquid extracts, powders, liposomal formulations, or nano emulsions. Polyphenol supplements offer higher bioavailability, standardized dosage, and convenience, eliminating the need for large food consumption. They provide targeted health benefits, such as cardiovascular support, brain function enhancement, and skin protection. Advanced formulations improve solubility and absorption, ensuring better efficacy. Nutraceuticals are bioactive compounds derived from plants, animals, fungi, yeast, and bacteria. While synthetic analogs of these natural compounds may be incorporated into future functional foods, plant-based nutraceuticals are often referred to as phytochemicals, sometimes interchangeably called medicinal botanicals. The distinction between nutraceuticals, dietary supplements, and pharmaceuticals is often blurred, leading to regulatory challenges. Since many pharmaceuticals originate from plant-derived compounds, addressing these overlaps remains a priority for regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (Wildman, Wildman, and Wallace 2016). Nutraceuticals, positioned at the intersection of pharmaceuticals and food, face hurdles in validating safety and health claims (Shekhar, Jha, and Dangi 2014). Unlike pharmaceuticals, which consist of high-dose, single-target purified compounds, nutraceuticals are complex mixtures that act on multiple pathways at lower concentrations. The terminology surrounding nutraceuticals, functional foods, medical foods, and dietary supplements remains ambiguous, with these terms frequently used interchangeably (Zhao 2007). Despite ongoing debates among experts regarding the precise definition of nutraceuticals, their expansion in the global market remains relentless. The rapid growth of this industry is largely driven by the absence of stringent regulatory frameworks, allowing for an increasing market presence. The proven benefits of nutraceuticals in promoting health and preventing disease, as endorsed by nutritionists and healthcare professionals, have fueled their worldwide demand. With growing consumer awareness and easy access to information on health-enhancing foods and supplements, billions of dollars are spent annually on these products. However, the global nutraceutical market remains a subject of ongoing discussion and regulatory scrutiny. This industry encompasses three primary sectors:

functional foods, natural or herbal products, and dietary supplements (Brower 1998) (Helal et al., 2019).

1.2 Dietary supplement Industry and Market

In recent years, the nutraceutical sector has experienced remarkable growth. The term "nutraceutical" merges the concepts of nutrition and pharmaceuticals, referring to products, largely of natural origin, that contribute to overall health. According to Nutraceutical World, global trade in the nutraceutical industry has been on an upward trajectory, with the COVID-19 pandemic serving as a significant catalyst. The heightened awareness of immune health led to a surge in demand for dietary supplements. However, this rapid growth also exposed vulnerabilities in global supply chain networks, forcing businesses to adapt swiftly to meet consumer needs. The nutraceutical and pharmaceutical companies worldwide are now shifting the focus on increasing the market trades of the bioactive as represented in the Figure 1 (Grand View Research 2018; Nutraceutical World 2023)

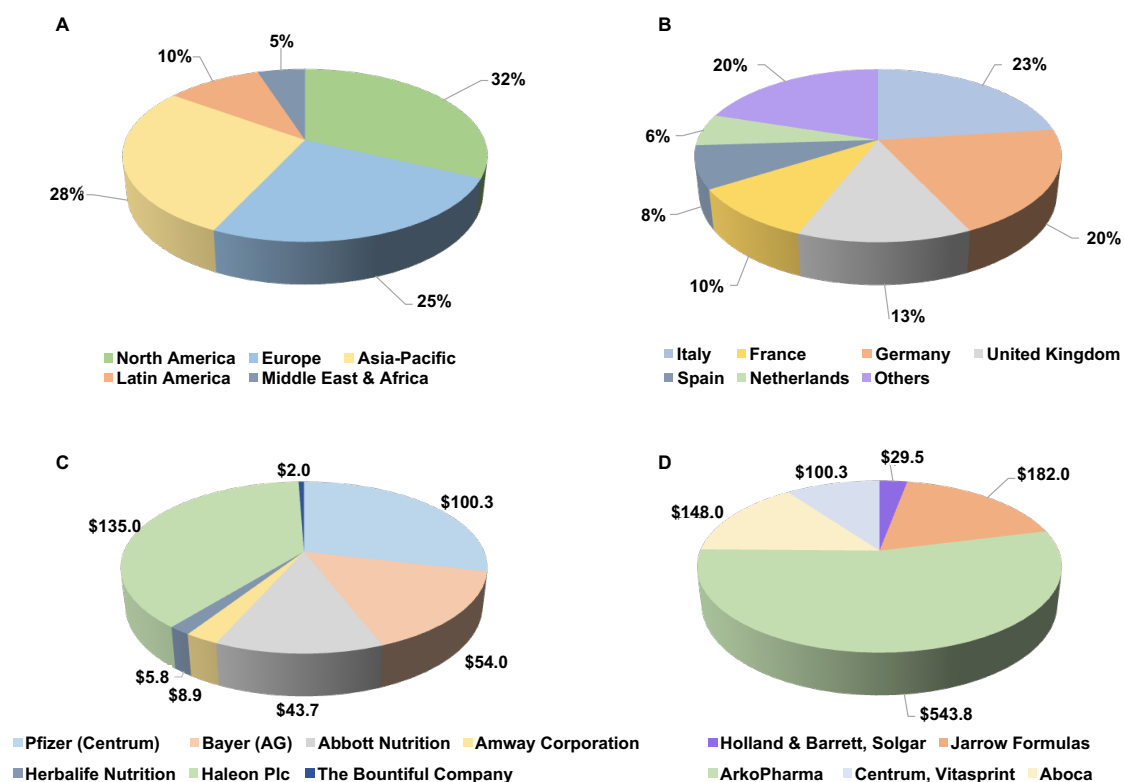


Figure 1 - Market Size of Nutraceutical Companies: (A) Global market size; (B) European market size; (C) Annual growth of nutraceutical companies worldwide; (D) Annual growth in Europe. All values are expressed in billions of dollars for 2022. (Grand View Research 2018; Nutraceutical World 2023)

The global nutraceutical market has witnessed a remarkable surge in recent years, driven by evolving consumer preferences, scientific advancements, and an increasing focus on preventive healthcare. The rising prevalence of lifestyle-related diseases, such as obesity, diabetes, cardiovascular disorders, and metabolic syndromes, has prompted individuals to

seek alternative solutions beyond conventional medicine. As a result, nutraceuticals, which offer health benefits beyond basic nutrition, have become an essential part of modern dietary patterns, particularly in the European and North American markets (Reque and Brandelli 2021). Consumers are becoming more conscious of the role that diet plays in overall well-being, leading to a significant shift towards functional foods, beverages, and dietary supplements. One of the primary factors contributing to the growth of the nutraceutical market is the increasing consumer preference for natural, plant-based, and functional ingredients (Télessy 2019). The modern-day emphasis on wellness, longevity, and disease prevention has led to a surge in demand for products enriched with bioactive compounds, including antioxidants, probiotics, polyphenols, and omega-3 fatty acids. The growing skepticism towards synthetic additives and pharmaceutical interventions has further propelled interest in nutraceuticals, as consumers seek holistic and sustainable health solutions. Furthermore, as awareness of personalized nutrition increases, there is a growing demand for targeted nutraceutical formulations designed to address specific health concerns, such as immune support, cognitive function, joint health, and digestive wellness (Speranza et al. 2017). Advancements in food and pharmaceutical sciences have also played a crucial role in the expansion of the nutraceutical sector. Cutting-edge research in biotechnology, nanotechnology, and food processing has enabled the development of more effective formulations with enhanced bioavailability, stability, and absorption. Traditional challenges, such as poor solubility and degradation of bioactive compounds, are being addressed through novel delivery systems, including liposomal encapsulation and microencapsulation techniques. These innovations ensure that nutraceuticals maintain their efficacy while offering improved shelf life and consumer convenience. Another driving force behind the growth of the nutraceutical industry is the aging global population. With increasing life expectancy, there is a heightened focus on maintaining health, preventing age-related diseases, and improving the quality of life. Older adults are actively seeking functional foods and supplements to support mobility, cognitive function, and cardiovascular health, further contributing to the expansion of the market. Additionally, the younger generation is also showing a strong inclination toward preventive health practices, fueled by digital accessibility to health-related information, social media influence, and a cultural shift toward proactive well-being (Đorđević et al. 2015; Timilsena et al. 2019; Zarrabi et al. 2020). The regulatory landscape has also evolved in response to the growing demand for nutraceuticals. Governments and health authorities worldwide are recognizing the potential benefits of functional foods and dietary supplements in reducing the burden on healthcare systems. Consequently, supportive regulatory frameworks are emerging, promoting product innovation while ensuring consumer safety. The European market, for instance, adheres to stringent regulations under the European Food Safety Authority (EFSA), ensuring that nutraceuticals meet high-quality standards and provide

scientifically validated health claims. Such regulations contribute to consumer trust and further bolster market growth (Santini et al. 2018). Globalization and urbanization have significantly influenced dietary habits, creating a wider acceptance of nutraceuticals in everyday consumption. With busy lifestyles and increased stress levels, consumers are seeking convenient and effective solutions to meet their nutritional needs. The demand for clean-label, non-GMO, organic, and sustainably sourced nutraceutical products is also on the rise, reflecting the emphasis on consumer for transparency and ethical consumption. The expansion of e-commerce and digital retail platforms has further fueled the accessibility of nutraceuticals, allowing consumers to explore a diverse range of products tailored to their individual health goals. Direct-to-consumer brands, subscription-based models, and personalized recommendations based on genetic and metabolic profiling are reshaping the industry. The convenience of online purchasing, coupled with the availability of expert reviews and consumer feedback, has empowered individuals to make informed choices, accelerating market penetration (Ren et al. 2021). Although Europe currently holds the largest share of the global nutraceutical market, the Asian market is experiencing the most rapid growth. Countries such as China, India, and Japan are witnessing a surge in nutraceutical consumption, driven by rising disposable income, increasing health awareness, and traditional practices that emphasize natural remedies. The incorporation of Ayurvedic and traditional Chinese medicine ingredients into modern nutraceutical formulations has further strengthened the market appeal in these regions. Additionally, government initiatives promoting functional foods and fortified nutrition to combat malnutrition and lifestyle disorders are further driving industry growth (Santini, Supino, and Bailetti 2023).

The future of the nutraceutical market appears promising, with continuous research and innovation shaping its trajectory. As the boundaries between pharmaceuticals, dietary supplements, and personalized nutrition continue to blur, the industry is set to play a pivotal role in the future of global health. With increasing consumer awareness, technological advancements, and regulatory support, the demand for nutraceuticals is expected to remain on an upward trajectory, reinforcing their significance in modern healthcare and dietary trends.

1.3 Plant based Supplements

Phytochemicals are biologically active compounds synthesized and accumulated in various plant parts, including roots, stems, flowers, fruits, and seeds. These compounds play a crucial role in protecting plants from diseases and environmental stress. Plant-derived nutraceuticals contain a diverse array of bioactive molecules that exert systemic effects by interacting with multiple biological targets across different organs. Their primary health benefits stem from their antioxidant, anti-inflammatory, antiproliferative, antimicrobial, and hypocholesterolaemia properties (Zhang et al. 2022). Bioactive phytochemicals can be classified into three main

categories: terpenes and terpenoids, phenolic compounds, and alkaloids. These compounds, extracted from various plant-based foods, regulate metabolic functions, and contribute to overall health. Some well-known bioactive compounds include carotenoids (e.g., γ -carotene, β -carotene, lutein, and lycopene), tocopherols (e.g., α -tocotrienol, γ -tocopherol, and γ -tocotrienol), phytosterols (e.g., β -sitosterol, stigmasterol, and campesterol), phenolic acids (e.g., cinnamic and benzoic acids), flavonoids (e.g., anthocyanidins, flavan-3-ols, flavones, flavanones, flavanols, and isoflavonoids), and saponins (e.g., soyasaponins). These bioactive compounds have been linked to the prevention of various chronic diseases, including cardiovascular disorders, neurodegeneration, and cancer. Among these, polyphenols are considered the most significant group of bioactive compounds found in plants, ranking second only to terpenoids in abundance (Vidal-Casanella et al. 2021). They are well known for their strong antioxidant potential, primarily attributed to their hydroxyl functional groups, which enable them to neutralize reactive oxygen species (ROS) and free radicals. The body naturally produces ROS and free radicals during metabolic processes; however, excessive accumulation leads to cellular damage by oxidizing nucleic acids, proteins, and membrane lipids. This oxidative stress has been closely linked to the development of numerous diseases. Antioxidant capabilities of polyphenols contribute to reducing the risk of conditions such as cancer, cardiovascular diseases, neurodegenerative disorders, diabetes, and obesity. Due to their beneficial properties, polyphenol-rich products have gained popularity as dietary supplements and nutraceuticals. Some of the most well-known polyphenol sources include tea, coffee, cocoa, wine, beer, nuts, fruits, vegetables, and aromatic herbs. Flavonoids represent the most abundant subclass of polyphenols, accounting for approximately 60% of total dietary polyphenols. Structurally, flavonoids possess a C6-C3-C6 backbone, composed of two aromatic rings connected by a three-carbon oxygenated heterocycle (Taroncher et al. 2021). These components are classified into six major classes mention in Figure 2. Among these, flavonoids have gained particular attention due to their versatile health benefits.

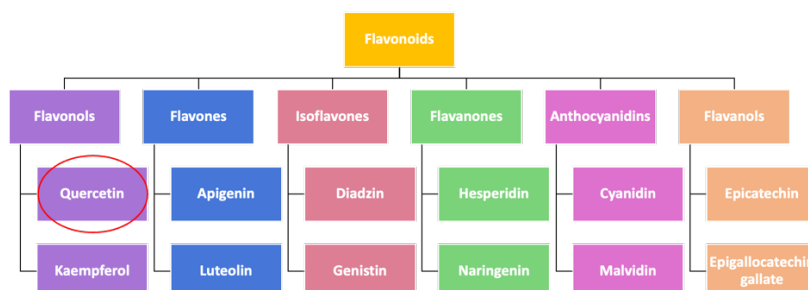


Figure 2 - Classification of Flavonoid. This figure categorizes flavonoids based on their chemical structure into major groups—flavones, flavanols, flavanones, isoflavones, anthocyanidins, and flavanols—highlighting bioactive components depending on the structural variation. (Taroncher et al. 2021).

Quercetin, a flavonoid, has been identified as one of the most potent dietary supplements. Many companies are shifting towards quercetin due to its strong market potential in the nutraceutical and pharmaceutical industries, driven by growing consumer demand for natural antioxidants, immune boosters, and anti-inflammatory agents. Figure 3 represent the data obtained from *Future Market Insights* on various dietary supplements and among them the market value of quercetin is being predicted higher than the other supplements (Future Market Insights Inc. 2022).

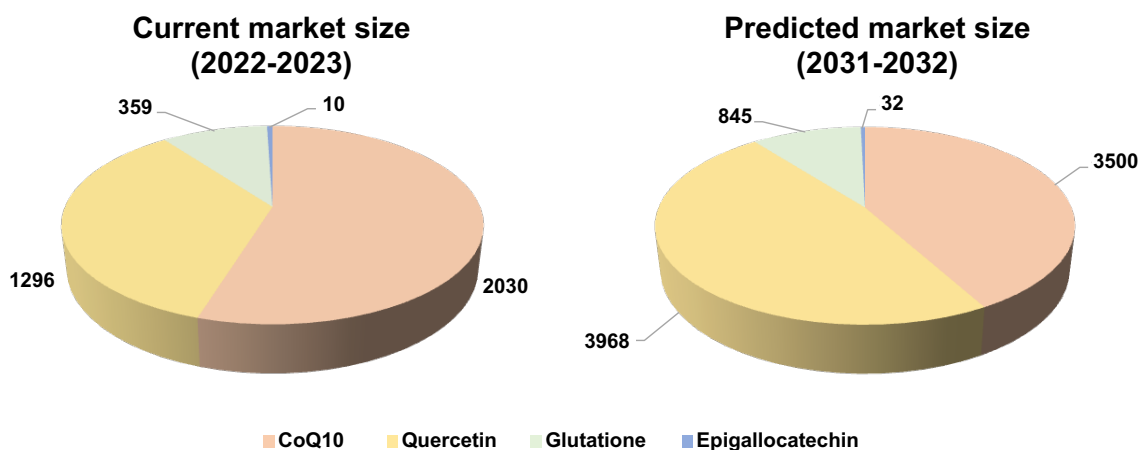


Figure 3 - Worldwide Market size of dietary supplements. The graphs show the comparison between the current market size (2022-2023) and the predicted marked size (2031-2032) in millions of dollars. (Future Market Insights Inc. 2022).

1.4 Quercetin: Multifaceted health effects

Quercetin, scientifically recognized as 3,3',4',5,7-pentahydroxyflavone, is a naturally occurring flavonoid distinguished by five hydroxyl groups located at positions 3, 5, 7, 3', and 4' in its molecular structure. It is also referred to as 3,3',4',5,7-pentahydroxy-2-phenylchromen-4-one. This bioactive compound is prevalent in a variety of plant-based foods and exists mainly in the form of glycosides. Quercetin consists of two benzene rings, labeled A and B, connected by a three-carbon heterocyclic pyrone ring. Due to the presence of two antioxidant pharmacophores in its structure, quercetin exhibits strong free radical-scavenging properties and can interact with transition metal ions. Additionally, the catechol moiety and the hydroxyl group at the C3 position make it highly effective in neutralizing free radicals. Structurally, quercetin is a penta-hydroxy flavonol, featuring five hydroxyl groups at the 3, 3', 4', 5, and 7 carbon positions in the Figure 4 (Vafadar et al. 2020; Williamson et al. 2000)

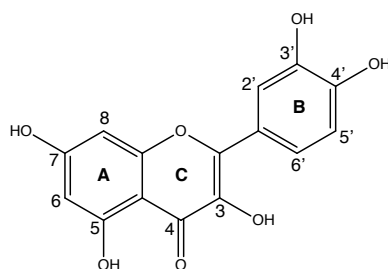


Figure 4 - Chemical structure of Quercetin (ChemDraw 16.0). This diagram illustrates the typical three-ring flavonoid backbone (rings A, B, and C) with standard numbering of substituent positions.

The introduction of different functional groups to its core structure can significantly influence its biochemical and pharmacological properties. Antioxidants play a crucial role in maintaining the balance of the body by neutralizing free radicals and reactive species that can cause cellular damage. These reactive molecules, such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), are formed naturally during metabolic processes. While some of these species, like hydrogen peroxide and nitric oxide, have physiological roles in immune response and cell signaling, others, such as hydroxyl radicals and peroxynitrite, can be highly destructive if their levels become excessive (Auten 2009; Schnabel et al. 2006). An imbalance between the production of these reactive species and the ability of the body to neutralize them leads to oxidative stress, a condition linked to aging and various diseases, including cardiovascular disorders, neurodegenerative diseases, and cancer. Although oxidative stress is often associated with harmful effects, reactive species also play beneficial roles in biological systems. They contribute to immune defense by helping immune cells destroy pathogens and support cellular signaling pathways that regulate functions like muscle relaxation and detoxification (Bast, Haenen, and Doelman 1991). However, when reactive species accumulate excessively, they can damage DNA, proteins, and lipids, leading to inflammation and cell death. This damage has been implicated in numerous chronic diseases, emphasizing the importance of a well-functioning antioxidant system (Diplock et al. 1998). To counteract the harmful effects of oxidative stress, the body relies on a defense system composed of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, work by converting reactive species into less harmful molecules. Non-enzymatic antioxidants, including vitamin C, vitamin E, and flavonoids, scavenge free radicals directly, preventing them from damaging cellular components. Its molecular structure allows it to effectively scavenge ROS and RNS, particularly superoxide anions and peroxynitrite, thereby reducing oxidative stress and inflammation (Halliwell and Gutteridge 2015; Rice-Evans and Miller 1996). In addition to its direct antioxidant effects, quercetin can enhance natural defense of the body mechanisms by increasing total plasma antioxidant capacity, further supporting its protective role against oxidative stress. Beyond its role as an antioxidant, quercetin exhibits strong anti-inflammatory

properties by modulating key signaling pathways involved in inflammation. It has been shown to inhibit the activation of nuclear factor kappa B (NF- κ B), a transcription factor that regulates the production of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) and interleukin-8 (IL-8). Quercetin effectively reduces cytokine-mediated inflammation by preventing the degradation of I κ B α which is an inhibitor of NF- κ B, which is particularly beneficial in conditions associated with chronic inflammatory responses, such as cardiovascular diseases and autoimmune disorders (MacNee 2001). In clinical studies, quercetin supplementation has been found to lower blood pressure in hypertensive patients, likely due to its ability to modulate oxidative stress and inflammation. Similarly, in patients with sarcoidosis, a chronic inflammatory lung disease, quercetin supplementation improved antioxidant capacity and reduced inflammatory markers, making it a potential therapeutic agent (Duthie et al. 2006; Perez-Vizcaino et al. 2006).

Table 1 - Various therapeutic effects of quercetin.

Disease	Dose	Function	Reference
Chronic obstructive pulmonary disease	1000 mg/day	Antioxidant, Platelet aggregation inhibitors, Prostaglandin synthase stimulant.	(Cialdella-Kam et al. 2013)
Pre- hypertensive and stage 1 hypertensive	4 weeks, 730 mg/day	Reduction of BP, no effects on oxidative stress parameters	(Edwards et al. 2007)
Cerebral oxidative injury	0.5-50 mg/kg (rodents)	Protect from oxidative stress and neurotoxicity	(Costa et al. 2016)
Anti-inflammatory	100 mg/kg (rodents)	release of cytokines: TNF- α and IL-1 β , NF- κ B pathway	(Comalada et al. 2005)
Breast cancer	10 mg/kg	Induced apoptotic cell death in BC cells via intensified combinatorial ROS(oxidative stress and mitochondrial damage)	(Xavier et al. 2011)
Colorectal cancer	25 mg/kg	Promoted apoptosis in KRAS-mutant CRC cells via activation of JNK signaling pathways and repression of the AKT pathway	(Yang et al. 2019)

1.5 Challenges in Bioavailability and Stability of Quercetin

Quercetin is a hydrophobic flavonoid with limited solubility in aqueous environments, which hinders its dissolution in gastrointestinal fluids and leads to poor absorption. In solution, it tends to aggregate into crystalline structures, further reducing its bioavailability (Cai et al. 2013). Even when a small portion dissolves, its low permeability ($5.5 \pm 0.3 \times 10^{-6} \text{ cm s}^{-1}$, cut off is $8 \times 10^{-6} \text{ cm s}^{-1}$) across the intestinal epithelium restricts passive diffusion. Once absorbed, quercetin undergoes extensive first-pass metabolism ($> 10\%$ bioavailability) in the liver and

intestines, where it is rapidly conjugated into glucuronides, sulfates, and methylated derivatives, often diminishing its biological activity (Hollman et al. 1997). The fast metabolic transformation and elimination through bile and urine result in a short half-life, necessitating frequent dosing to maintain effective levels. Stability is another challenge, as quercetin is prone to oxidative degradation and instability under physiological pH conditions, further reducing its systemic availability (Amorati et al. 2017). To address these limitations, various delivery systems have been explored such as liposomes, phytosomes, lipid nanoparticles, and nanocrystals (Puttasiddaiah et al. 2022). Liposomal encapsulation, particularly through microfluidic technology, enables precise control over vesicle size and uniformity. This method involves controlled mixing of lipid solutions with an aqueous phase under regulated flow conditions, ensuring reproducibility and scalability (Webb et al. 2019). Nanocrystals offer another promising approach to enhancing the solubility and dissolution rate of quercetin (Byrappa, Ohara, and Adschiri 2008). They can be produced through techniques such as supercritical CO₂ processing and solvent injection. The supercritical CO₂ method relies on the rapid expansion of a supercritical solution containing quercetin and a stabilizer, leading to the formation of nano-sized crystalline particles with improved dispersibility (Franco and De Marco 2021).

1.6 Supercritical Carbon Dioxide technique (Sc-CO₂) - A Sustainable Approach

Prior to understanding the supercritical CO₂ fluid, at standard conditions, carbon dioxide (CO₂) exists as a gas; however, at standard temperature and pressures exceeding 3.5 MPa, it transitions into a liquid state. When subjected to temperatures above 304.13 K and pressures beyond 7.4 MPa, CO₂ enters its supercritical phase (Nikolai et al. 2019). The carbon dioxide molecule is generally considered non-polar or at least non-dipolar. Each C=O bond in CO₂ has a length of 116.21 pm (as per NIST data), leading to a significant separation of opposite charges and an inherent dipole moment within each bond. However, due to the linear geometry of the molecule, the total dipole moment cancels out as the two-opposing partial-moment vectors nullify each other. Despite this, at a molecular scale, these dipole moments contribute to a quadrupole moment, which enhances solvation ability of CO₂ through quadrupole–dipole and quadrupole-induced dipole interactions (Calcara and Caricaterra 2023). Carbon dioxide typically acts as a Lewis acid or base, with its carbon atom binding nucleophiles and its oxygen atoms interacting with electrophiles. In self-coordination, this Lewis acid/base mechanism manifests as an interaction between the lone pair of oxygen from one CO₂ molecule and the electron-deficient carbon of another (Raveendran and Wallen 2002). CO₂ predominantly exists in two dimeric forms: the slipped-parallel and the T-shaped configurations as represented in Figure 5. In the slipped-parallel structure, a weak hydrogen bond forms between the central carbon atom of one molecule and an oxygen atom of the

adjacent molecule, while the carbon of the second molecule simultaneously interacts with the second oxygen of the first. This configuration is most prevalent in the gaseous phase but decreases in occurrence as pressure increases, becoming less significant in the liquid and supercritical states, where the T-shaped configuration becomes dominant (Di Noto et al. 2011; Saharay and Balasubramanian 2004; Zhang, Yang, and Yu 2005). In the gaseous phase, due to low density, anisotropy is observed, whereas in the liquid and supercritical states, anisotropy diminishes, leading to a more ordered structural arrangement (Cipriani et al. 2001).

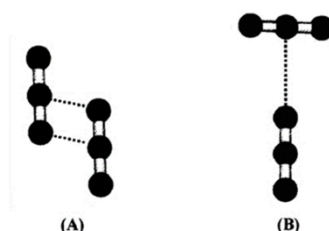


Figure 5 - Configuration of CO₂ (A) Slipped-parallel, (B) T-shaped configuration representing the dimeric forms (Zhang et al. 2005).

In the supercritical region, the P-V isotherm exhibits a remarkably small slope, indicating that even minor adjustments in pressure can lead to substantial volume changes. This high isothermal compressibility distinguishes SCFs from conventional fluids, where compressibility is generally much lower at higher pressures. In the P-V-T phase diagram represented in the Figure 6 below gives an overview of the supercritical fluids (SCFs) other than CO₂. The transition to the supercritical state is observed above the critical point (C.P.) on both P-T (pressure-temperature) and P-V (pressure-volume) projections.

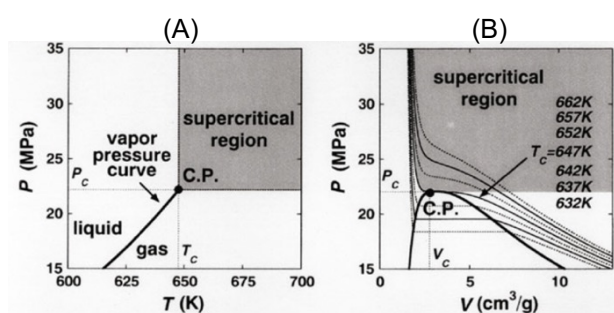


Figure 6 - Supercritical fluids isotherm (A) P-T curve and (B) P-V curve representing the transition state to supercritical region above the critical point (Sako 2002).

The critical properties of SCFs vary based on the molecular composition of the fluid, with key parameters such as critical temperature (T_c), critical pressure (P_c), and compressibility factor (Z_c) influencing their behavior. The critical properties of SCFs vary based on the molecular composition of the fluid, with key parameters such as critical temperature (T_c), critical pressure

(Pc), and compressibility factor (Zc) influencing their behavior. As summarized in table 2, relatively simple molecules such as argon (Ar), methane (CH₄), and xenon (Xe) exhibit critical temperatures ranging from 151 K to 290 K, with compressibility factors between 0.235 and 0.296. CO₂ is one of the most used SCFs, has a critical temperature of 304 K and a critical pressure of 73 atm, making it an ideal candidate for various industrial and pharmaceutical applications due to its moderate supercritical operating conditions. In contrast, water (H₂O) requires significantly higher pressures (218 atm) and temperatures (647 K) to reach its supercritical phase, making it less accessible for certain applications (Sako 2002).

Table 2 - Critical Temperature and Pressure of different solvents showing their supercritical state (Sako 2002).

Solvent	Critical Temperature (°C)	Critical Pressure (Bar)
Argon	-122	49
Methane	-82	45
Xenon	17	58
Trifluoromethane	26	47
Carbon Dioxide	31	74
Ethane	32	48
Nitrous Oxide	34	71.5
Ammonia	133	111
Ethanol	241	61.4
Water	374	218

One of the most prominent applications of supercritical carbon dioxide (SC-CO₂) is in supercritical fluid extraction (SFE), where its solvent strength can be enhanced by introducing co-solvents such as ethanol or methanol. These co-solvents modify the polarity and solvent strength of SC-CO₂, allowing for the selective dissolution of a broader range of compounds. A small amount (typically 3–5 mol%) of a co-solvent can significantly improve solubility and selectivity, making SC-CO₂ an effective medium for processes such as caffeine removal from coffee beans, bioactive compound extraction, and polymer impregnation (Machado et al. 2013). SC-CO₂, other than the extraction, it is applicable also for the chromatography, catalysis, and pharmaceutical formulation development (Knez et al. 2014).

1.7 Supercritical CO₂ Technology Methods

There are several ScCO₂-based techniques classified based on the role of CO₂ in the process: as a solvent, an antisolvent, or a co-solute. When CO₂ is used as a solvent, the process involves dissolving the active pharmaceutical ingredient (API) in supercritical CO₂, followed by rapid expansion, leading to precipitation. The Rapid Expansion of Supercritical Solutions (RESS) technique is one such method, where the API is dissolved in ScCO₂ and rapidly

depressurized through a nozzle, leading to particle precipitation. Modifications such as RESS-SC (Solid Co-solvent) and RESOLV (Rapid Expansion of Supercritical Solution into a Liquid Solvent) enhance drug solubility and allow the precipitation of finer particles (Machado et al. 2013). Alternatively, ScCO_2 can act as an antisolvent, where the drug is first dissolved in an organic solvent, and then ScCO_2 is introduced to precipitate the drug by reducing solubility. This approach includes techniques such as Supercritical Antisolvent Precipitation (SAS), Aerosol Solvent Extraction System (ASES), and Solution-Enhanced Dispersion by Supercritical Fluids (SEDS) (Fraile et al. 2014). These methods allow precise control over particle size, morphology, and crystallinity, making them particularly useful for poorly water-soluble drugs. Furthermore, Sc-CO_2 can function as a co-solute, where it dissolves in a liquid solvent containing the drug, modifying its properties and enhancing precipitation. This approach is commonly used in Particles from Gas-Saturated Solutions (PGSS), where ScCO_2 is absorbed into a melted drug-polymer mixture before being rapidly expanded to form nanoparticles (Chafidz et al. 2018).

In the presented work with Supercritical CO_2 and Liquid CO_2 the quercetin complexes were prepared using the polymers namely Pluronic F 127 and 2-HP- β cyclodextrin in ethanol as a cosolvent using SAS technique.

1.8 Properties of Poloxamer 407- Pluronic F 127

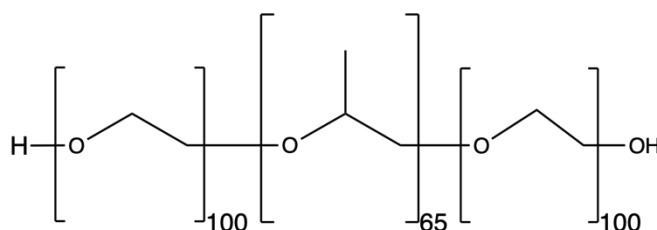


Figure 7 - Molecular structure of Pluronic F 127 (ChemDraw 16.0).

The Pluronic F 127 (M.W. 12,600 g/mol) is a triblock copolymer of polyethylene oxide (PEO) and polypropylene oxide (PPO) which are often denoted as $(\text{PEO})_{100}$ -(PPO) $_{65}$ -(PEO) $_{100}$ units represented in the Figure 7 (Alexandridis and Hatton 1995). It contains OH group at both the ends allowing the formation of hydrogen bond with the ethanol, supercritical CO_2 and liquid CO_2 meanwhile, the ether linkages between PEO and PPO found to participate in the dipole-dipole interactions with supercritical and liquid CO_2 via its quadruple dipole interactions (Danten, Tassaing, and Besnard 2002). At low concentrations and temperatures, the copolymer exists as individual unimers; however, as the concentration or temperature increases, micellization occurs due to the hydrophobic effect, wherein PPO segments aggregate to minimize their exposure to water, while PEO chains extend into the aqueous phase, stabilizing the micelles (Grillo, Morfin, and Prévost 2018). The critical micellization concentration (CMC) and critical micellization temperature (CMT) denote the transition point

where micelle formation begins. Both parameters decrease with increasing PPO content or molecular weight, indicating that more hydrophobic copolymers undergo micellization more readily. The process is endothermic, driven primarily by entropy gain as water molecules are released from the hydration shell of PPO chains upon aggregation (Dumortier et al. 2006). Pluronic F-127 also demonstrates thermoresponsive gelation at high concentrations, where the micelles organize into a structured network, forming a gel-like phase. The gelation onset temperature and thermal stability of the gel increase with the length of the PEO blocks, which influence hydration and intermolecular interactions (Juhasz et al. 1989). Pluronic F-127 highly valuable in applications such as drug delivery, solubilization of hydrophobic compounds, emulsification, and biomedical formulations, where controlled micellization and gelation behaviors are advantageous (Pluronic 2006).

1.9 Properties of Beta-cyclodextrins

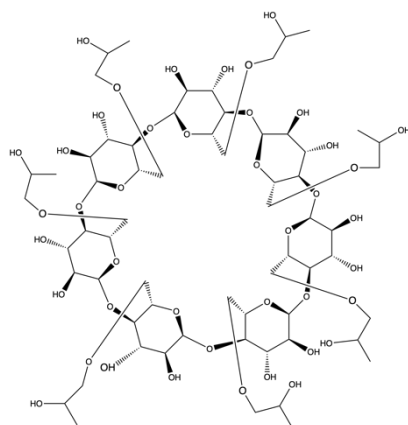


Figure 8 - Molecular structure of 2- HP betacyclodextrin (ChemDraw 16.0).

Beta-cyclodextrin (β -CD), a cyclic oligosaccharide composed of seven glucose units linked by α -1,4-glycosidic bonds, possesses a unique truncated cone-like structure characterized by a hydrophobic inner cavity and a hydrophilic outer surface as shown in Figure 8. This distinctive molecular architecture enables β -CD to form non-covalent inclusion complexes with hydrophobic guest molecules, significantly enhancing their solubility, stability, and bioavailability (Szente and Szejtli 1999). Despite its versatile applications, native β -CD exhibits limited aqueous solubility (approximately 18.5 mg/mL at 25°C) due to strong intermolecular hydrogen bonding, which has necessitated the development of chemically modified derivatives to improve its functional properties. The most significant derivatives include methylated cyclodextrins, hydroxypropyl cyclodextrins, sulfobutylated cyclodextrins, and sulfated cyclodextrins, each exhibiting distinct physicochemical and biological properties. Among these, hydroxypropyl- β -cyclodextrin (HP- β -CD) demonstrates superior water solubility (>600 mg/mL) and is widely utilized in pharmaceutical formulations to facilitate drug solubilization, controlled release, and reduce toxicity making it a preferred excipient for oral formulations (Fourmentin, Crini, and Lichtfouse 2018). Studies have demonstrated that HP- β -

CD not only enhances the solubility and dissolution rate of poorly soluble flavonoids but also stabilizes them against enzymatic and chemical degradation within the gastrointestinal tract (Yamamoto et al. 1989). In an in vivo absorption study conducted on beagle dogs, oral administration of a rutin-HP- β -CD complex resulted in a significantly higher plasma concentration of homovanillic acid (HVA), a major metabolite of rutin, compared to rutin alone or its β -CD complex (Miyake et al. 2000). The increased oral bioavailability of the active in the presence of HP- β -CD is attributed to its superior solubilization capacity, improved dissolution kinetics, and enhanced gastrointestinal stability, preventing premature degradation before systemic absorption (Salústio et al. 2011).

2. Aim of Work

This study set out to develop advanced delivery systems that enhance the solubility, stability, and bioavailability of the compound. A trio of formulation strategies—supercritical CO₂ (Sc-CO₂), liquid CO₂, and the conventional solvent injection method—were investigated to produce quercetin nanocrystals. Underpinning this effort, the Solvent Antisolvent (SAS) technique was applied to prepare quercetin with Pluronic F127 and 2-hydroxypropyl β -cyclodextrin using Sc-CO₂, while liquid CO₂ was similarly employed to scrutinize the impact of different CO₂ states on yield, morphology, and molecular interactions. The extensive literature research was done for selecting the most suitable co-solvents for either supercritical or liquid CO₂. A comparative study with traditional solvent injection methods provided insight into the distinct characteristics of each formulation. Analytical techniques including XRD, FTIR, DSC, DLS, and TEM illuminated the structural and physicochemical nuances of the resulting nanocrystals, while drug release studies performed under the simulated gastric and intestinal conditions revealed their behaviour in physiological settings. Finally, antioxidant assays in oxidant-stressed healthy cells confirmed that these nanocrystals effectively reduced reactive oxygen species (ROS), underlining their promising functional benefits and positioning them as potent candidates for diverse therapeutic applications.

3. Materials and Methods

3.1 Materials

Quercetin dihydrate (>95% purity, MW: 338.32 g/mol) was used as an active compound provided by Farmalabor SRL. Ethylene oxide– propylene oxide block copolymer Pluronic F 127 (MW: 12,600 g/mol), 2-Hydroxypropyl- β -cyclodextrin (MW: 1396), Ethanol absolute, acetone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbon dioxide (CO₂) was used as an antisolvent. Fasted state simulated intestinal fluid (FaSSIF) pH 6.5 without Enzyme Dissolution Media was purchased from Reagecon. Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were purchased from Sigma Aldrich. Water was purified with Milli-Q® (Millipak® 0.22 μ m) system.

3.2 Preparation of the Quercetin microcrystals and Quercetin/Pluronic F127 crystals using Supercritical CO₂ as an antisolvent

The Supercritical Anti-Solvent (SAS) method was employed to prepare quercetin microcrystals and crystals of Quercetin/Pluronic F127. The experiments were conducted using a laboratory apparatus equipped with a Jasco PU-2080-CO₂ Plus delivery pump, which generated supercritical CO₂ that was introduced into a 50 mL reactor vessel via a coaxial nozzle. A separate pressure pump (Jasco PU-2080) was dedicated to delivering ethanol as the cosolvent into the reaction vessel. Stainless steel tubing connected both the supercritical CO₂ pump and the ethanol pump to the reactor vessel, which was heated by a Chrompack CP-9003 oven to maintain supercritical CO₂ conditions at 40°C. The system pressure was regulated using a Jasco 2080 Plus Automatic Back Pressure Regulator. The pressure and temperature were carefully controlled to optimize the conditions for nanocrystal formation. For the preparation of quercetin microcrystal, pure quercetin solution (10 mg/mL) was prepared by dissolving in ethanol. For the preparation of Quercetin/Pluronic F127 crystals, each of the component was dissolved in ethanol with the molar ratio of 1:1 having 2 mg/mL each. Both total solution prepared was of 45 mL. Supercritical CO₂ (Sc-CO₂) and the respective ethanol solutions were simultaneously pumped into the reactor vessel through the coaxial nozzle at varying flow rates and pressures. Pure quercetin microcrystal was obtained within 3 hours of preparation meanwhile precipitation of quercetin-Pluronic F127 occurred within two hours of processing. At the end of each experiment, pure supercritical CO₂ was pumped through the system for 40 minutes to remove residual organic solvents. The reactor vessel was then opened, and the recovered crystals were dried in a hot air oven at 40°C until completely dry.

3.3 Preparation of Quercetin/2-Hydroxypropyl- β -cyclodextrin crystals using Supercritical CO₂ technology

The Quercetin (3.65 mg/mL) and 2-Hydroxypropyl- β -cyclodextrin (13.74 mg/mL) were dissolved in the ethanol (30 mL) with 1:1 molar ratio. Supercritical CO₂ and the respective ethanol solutions were simultaneously pumped into the reactor vessel through the coaxial nozzle. However, no precipitate formation was observed for quercetin-2-hydroxypropyl- β -cyclodextrin, even when varying pressure and flow rate conditions and time also specified in table 3 of Results and Discussion

3.4 Preparation of quercetin/Pluronic F127 and quercetin/2-HP- β -cyclodextrin crystals using Liquid CO₂ as an antisolvent

For these experiments, a 115 mL iron autoclave equipped with a manometer was employed. The autoclave was charged with 30 mL of the ethanol solution containing quercetin (2.03 mg/mL) and PF127 (75 mg/mL) owing to 1:1 molar ratio. The quercetin (4.06 mg/mL) and 2-HP- β -cyclodextrin (16.75 mg/mL) were similarly prepared maintaining the 1:1 molar ratio which were dissolved in ethanol (30 mL). The solutions were pressurized to 100 bars with CO₂ before being placed in an ice-salt bath maintaining 0°C temperature. The liquid CO₂ was delivered at flow rate of 2 mL/min for 2 hours. The solutions were placed inside a cylindrical Dewar vessel. The precipitated crystals were then recovered and subjected to drying using a rotary evaporator.

3.5 Preparation of crystals using Solvent injection method (batch method)

In the solvent evaporation method, quercetin was dissolved in 20 mL of ethanol, while 2-HP- β -cyclodextrin and PF127 was dispersed in 60 mL of distilled water with the similar 1:1 molar ratio. The two solutions were combined and stirred continuously for one hour to ensure thorough mixing. Ethanol was subsequently removed by heating at 40°C under constant stirring at 500 rpm. The remaining aqueous phase was then eliminated by drying the mixture in a hot air oven at 40°C for 24 hours. Once fully dried, the crystals were finely ground using a mortar and pestle, weighed to determine the final dry mass, and stored in an airtight container for further analysis.

3.6 Phase Solubility studies

The phase solubility study was carried by the Higuchi and Connors method (Saokham et al. 2018). An excess amount of QUR (0.1g) was added to 5 mL distilled water in which various concentration of β -CD (0, 2, 4, 6, 8, 10, 15, 20 mM) was added. The flask was then shaken continuously at 37°C for 48 hours. Samples of 3 mL were withdrawn and filtered through Whatman filter paper and then diluted up to 200 times with absolute ethanol to determine the quercetin concentration and assayed at 370 nm (UV Cary). By using phase solubility diagram, stability constant (K_{st}) was calculated using equation:

$$K_{st} = \frac{Slope}{S_0 (1 - slope)}$$

The slope was being obtained from the plot of Quercetin concentration against β -CD concentration. Where, k_{st} is stability constant, S_0 is the intercept.

3.7 Dynamic Light Scattering (DLS)

The crystal size, uniformity and zeta potential were examined by Zetasizer Nano particle analyser (Malvern Panalytical, Malvern, UK). The size was measured at 25 degs, equilibrating time of 60 seconds. The samples were diluted from 1:10 to 1:100 to optimize the desired dilution for the estimation.

3.8 Transmission Electron Microscopy (TEM)

A 25 μ L drop of the solution was placed onto a 400-mesh holey film grid. Following a 2-minute staining with 1% uranyl acetate, the sample was examined using a FEI Tecnai G2 transmission electron microscope (Hillsboro, Oregon, US) operating at 100 kV. Images were captured with a Veleta digital camera (Olympus Soft Imaging System) and processed using ImageJ software.

3.9 Drug loading Efficiency

Quercetin/Pluronic F127 and Quercetin/2HP- β -Cyclodextrin crystals, prepared using the liquid CO_2 and solvent injection method, were dissolved in absolute ethanol to facilitate the extraction of quercetin. The ethanol solutions were then filtered through 0.45 μ m filters to obtain purified quercetin solutions. The quercetin content was quantified at 370 nm using a UV-Vis spectrophotometer (CARY UV) The drug loading efficiency was calculated using the following equation:

$$\% \text{ Drug loading} = \frac{\text{Amount of Quercetin encapsulated}}{\text{Total inclusion complex}} \times 100$$

3.10 Fourier Transform- Infrared Spectroscopy (FTIR)

FT-IR spectroscopy was performed on the powdered nanocrystals to ascertain their molecular structure. KBr disks were prepared using the powder samples, and their FTIR spectra at resolution of 2 cm^{-1} , 128 number of scans within the spectral range of 400-4500 cm^{-1} that was recorded in the absorbance mode of acquisition at ambient temperature using a Perkin Elmer-Spectrum One spectrophotometer.

3.11 Differential Scanning Microscopy (DSC)

The DSC thermogram pattern was recorded on the thermal analyzer. The mixture sample was heated in closed aluminum crimped cells at a heating rate of 10°C/in the 30-300°C temperature range under a nitrogen atmosphere at a flow of 50ml/min.

3.12 X-ray diffraction (XRD)

XRD was performed to study the crystalline nature of the formulations. Powdered nanocrystals were measured using powder specimen holder. Samples were analysed by XRD diffractometer Empyrean (Malvern Panalytical, Worcestershire, UK). CuK α radiation was used, the diffractograms were recorded by setting a diffracted intensity detection step equal to 0.05°. The unit of measurement of the detected intensity is the number of pulses / second. The XRD spectra reported the intensities of the diffracted X ray beams as a function of the angle.

3.13 In vitro drug release

In vitro release tests were performed in simulated gastric and intestinal fluids. Simulated gastrointestinal fluids were prepared according to the following procedures: in the case of gastric fluid, the pre-prepared FASSIF media was pH adjusted to 1.6 by adding HCl. In the case of intestinal fluid, the FASSIF media was used purchased from Reagecon. Pure quercetin, Quercetin/Pluronic F 127, Quercetin/2-HP-betacyclodextrin powdered prepared in Liquid CO₂ and batch method (1 mg) were dissolved in filtered 0.22 μ m millQ (1mL). The suspension was added in the dialysis bag (MWCO, 5000 Da) and was immersed in a beaker containing 43 mL of the release media. The conditions were maintained at 37 °C and stirred (100 rpm). After predefined periods of 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 mins samples were taken and replaced by the fresh simulated gastric/intestinal media. The release media were filtered through 0.45 μ m filters and the filtrates were measured with the UV-Vis spectrophotometer at 370 nm. The experiments were performed in triplicate.

3.14 Antioxidant analysis

MRC-5 cells (8×10^3 per well) were seeded in a 96-well plate and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate (NaPi), and 1% non-essential amino acids (NEAA), following the supplier's instructions. The cells were allowed to adhere overnight at 37°C in a humidified atmosphere with 5% CO₂. The following day, the cells were treated with 250 μ M H₂O₂, 50 μ M Quercetin, 50 μ M Quercetin-Bcd, 50 μ M Quercetin-P F127, or combinations of 50 μ M Quercetin samples with 250 μ M H₂O₂. After 6 and 24 hours of incubation, reactive oxygen species (ROS) production was evaluated using the ROS-Glo™ H₂O₂ Assay (Promega, cat. G8820) according to the manufacturer's instructions.

4. Results and Discussion

4.1 Ethanol as a cosolvent selection

Initially, the literature understanding between the solvent interaction and Sc-CO₂ was extensively done. Acetone and ethanol interact with liquid and supercritical CO₂ in distinct ways, primarily due to differences in their functional groups and binding mechanisms. Acetone, containing a carbonyl (C=O) group, forms stronger direct interactions with CO₂ through O=C $\cdots$ CO₂ dipole-quadrupole interactions, making it more energetically favorable in terms of binding energy (enthalpy, ΔH) compared to ethanol. The Lewis basicity of the carbonyl oxygen allows acetone to interact with the electrophilic carbon of CO₂, leading to more negative ΔH , which means a stronger and more exothermic interaction (Blatchford, Raveendran, and Wallen 2003). In contrast, ethanol, with its hydroxyl (-OH) group, forms weaker individual bonds with CO₂, primarily through hydrogen bonding (O-H $\cdots$ CO₂) and dipole-quadrupole interactions. However, ethanol compensates for its weaker direct binding by establishing multiple hydrogen bonds, leading to a cooperative solvation network that increases local CO₂ density and enhances overall solvation properties (Fulton, Yee, and Smith 1991; Saharay and Balasubramanian 2006). In the supercritical state of 100-200 bars and 40°C, acetone remains more dispersed in CO₂ due to its strong but localized interactions, while ethanol, despite weaker binding, contributes to stronger clustering effects, forming structured hydrogen-bonding networks that modify the solvent environment. This means that acetone is better for dissolving weakly polar and nonpolar compounds in supercritical CO₂, whereas ethanol plays a key role in altering CO₂ density and enhancing solvation capacity. At 100 bars and 0°C, where CO₂ is in its liquid phase, both ethanol and acetone exhibit stronger interactions due to higher molecular density, but ethanol experiences a larger entropy loss (ΔS more negative) as hydrogen bonding becomes more structured, further enhancing its role in solvent structuring. Acetone, on the other hand, benefits from the denser CO₂ environment, which strengthens its O=C $\cdots$ CO₂ interactions without a significant entropy penalty, maintaining greater solubility flexibility. Thus, while acetone forms stronger direct interactions with CO₂ in terms of binding energy, ethanol has a greater impact on overall solvation and solvent structuring due to its hydrogen bonding abilities. In liquid CO₂, ethanol enhances molecular organization, while in supercritical CO₂, it influences solvent density and clustering, making it useful for enhancing solvation properties. Acetone, however, remains the better solvent for weakly polar or nonpolar compounds, given its stronger direct interactions and lower entropy constraints (Kong, Funazukuri, and Kagei 2006; Nishiumi, Fujita, and Agou 1996; Saric, Guevara-Carrion, and Vrabec 2022; Sassi et al. 1987; Vaz, Gomes, and Silva 2016)

4.2 Micronization of Quercetin using Supercritical CO₂

Table 3 - Optimization parameters of Quercetin crystals using Supercritical CO₂ technique.

Formulation	C _i (mg/mL)	CO ₂ flow rate (mL/min)	Organic flow rate (mL/min)	FRR	Pressure (bar)	Run Time (Hours)	Amount Recovered (mg/mL)	Yield (%)	Loading (%)
QC_{Microcrystal}	10	5	0.3	16.7 :1	100	3	None		--
	10	5	0.06	83.3 :1	100	3			--
	10	2.5	0.06	41.6 :1	100	3	0.8	8%	--
Qc PF127_{Sc-CO2}	4 (1:2) mM	2.5	0.06	41:1	100	3	None		--
	4 (1:1) mM	2.5	0.15	16.7 :1	200	5	3.04	76%	30%
Qc βCD_{Sc-CO2}	9.95 (6:6) mM	2.5	0.15	16.7 :1	200	5	--	--	--
	9.95 (6:6) mM	1.5	--	--	200	2	--	--	--
	9.95 (6:6) mM	5	--	--	100	2	--	--	--
	18.9 (12 mM each)	5	--	--	100	2	--	--	--

To understand the behavior of supercritical CO₂ with quercetin molecules dissolved in ethanol, the experiment was performed at varied FRR between the CO₂/solvent keeping the pressure, temperature, and time constant. In the table 3, the failure in the micronization of the quercetin when the FRRs of CO₂/solvent could be possibly due to high relative flow rate of organic solvent (0.3 mL/min) to the CO₂ (5 mL/min) where an ethanol–quercetin solution may remain in too solvent-rich environment so quercetin couldn't exceed its solubility limit in the CO₂-expanded mixture thus never reaching the critical supersaturation required for nucleation and remain dissolved in the ethanol solution yielding no product. Conversely, when the organic solution drips in too slowly (0.06 mL/min) while the CO₂ sweeps aggressively (5 mL/min), quercetin can become diluted in a continuously expanding solvent phase and again fail to coalesce into solid particles. (Bertucco, Pallado, and Benedetti 1996) explains about the supercritical fluids-organic solvent phase separation results in the solute molecule (quercetin) to propagate towards to its likely ethanol environment. In the entire process, the importance of vessel height and diameter is an influential parameter as well. The high flow rate of CO₂ (5 mL/min) swirled in the low height to diameter vessel resulting in less residence time. Meanwhile, in the third condition the CO₂ flow rate was lowered (2.5 mL/min) while keeping the organic solution constant (0.06 mL/min) adjusting the ratio to moderate FRR of 41.7 allowing local supersaturation where quercetin is being gradually accumulating and forming the crystals with 8 % yield. The final yield was not high as expected because of the delayed

extraction due to the formation of larger particle size. In accordance with (Fiori, Basso, and Costa 2008) at a certain run time early stages of extraction may proceed quickly on the quercetin surface, but once that outer portion is depleted, the slower internal diffusion becomes the bottleneck. On the positive insight, Fiori et al. describes that the delayed extraction can be linked to stronger bonding (e.g., hydrogen bonding, Van der Waals forces) within the matrix. Larger particles often maintain more “intact” internal structures, meaning the solute is more strongly retained and not easily liberated which can also be observed in the TEM images Figure 9.

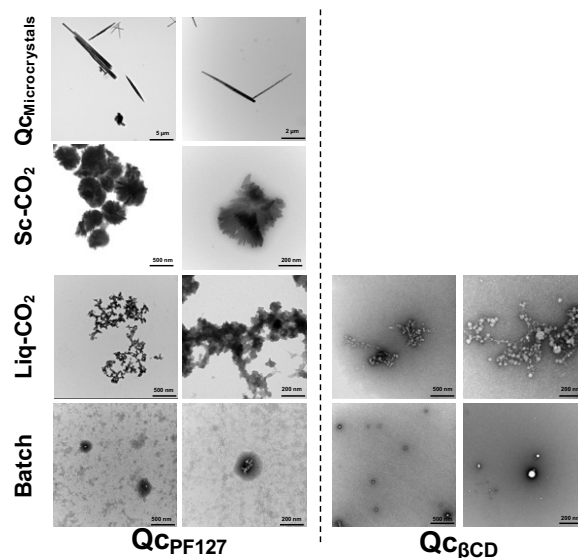


Figure 9 - TEM analysis of Quercetin formulations. Needle-shaped Qc_{microcrystals}, spherical nucleated Qc_{PF127} crystals prepared via supercritical CO₂, and agglomerates of Qc_{PF127} and Qc_{βCD} produced using liquid CO₂. The batch method shows spherical crystals with an outer micellated structure in Qc_{PF127} formulations, while Qc_{βCD} formulations display a well-defined cyclodextrin cavity layer.

4.3 Formation of Quercetin/Pluronic f 127 crystals using SAS method

Meanwhile, when the similar operating conditions were applied to prepare quercetin/pluronic f 127 crystals unfortunately lead to no precipitation but only the presence of the liquid layer at the bottom of the vessel was observed. In table 3, the pressure conditions 100 bars seem to impact less likely on the quercetin/pluronic f 127 ethanol solution. One key factor is that at 100 bars and 40 °C, the density of CO₂ is approximately 629 kg/m³, notably lower than the 840 kg/m³ at 200 bars, 40 °C based on (Peace Software, Berndt Wischnewski, Online Calculation-Carbon Dioxide). Near the critical point—73.8 bars and 31 °C—the CO₂ density is about 411.5 kg/m³. This 53% density increase (from near-critical to supercritical) prompts a complete phase transition that enables micronization of pure quercetin at 100 bars. When the pressure rises further to 200 bars, the CO₂ density gains another 34%, which may seem modest but nonetheless triggers non-linear effects on solubility and diffusivity making CO₂ more miscible with the quercetin/Pluronic F127-ethanol mixture, thereby enhancing the solvent extraction and facilitating particle formation as described in (Daode Hu 2012). In

reference to (Boutin and Badens 2009) the range of 0.01–0.2 mL/min are typically considered low for such small reactors—these conditions allow for gradual supersaturation and controlled solvent extraction, leading to finer, more uniform particles. In the experiment, the quercetin/Pluronic F127-ethanol flow rate used was 0.06 mL/min in the initial condition which is considered relatively (CO_2 flow rate 2.5 mL/min) too low for the 40 mL vessel resulting no burst supersaturation needed for rapid nucleation. According to the experimental evidence of (Boutin and Badens 2009) the flow rate of organic solution above 0.2 – 0.3 mL/min is considered higher and often cause rapid injection, sometimes leading to particle breakup and broader irregular particle size distribution and this also leads to incomplete particle precipitation resulting in low yield. Therefore, the operating parameters of organic solution flow rate of 0.15 mL/min in combination with CO_2 flow rate of 2.5 mL/min provided an optimum condition that effectively balanced the mass transfer and supersaturation dynamics necessary for high-yield crystallization of 76% with quercetin to polymer drug loading of 30 % (Table 5) with unimodal particle size distribution (Figure 10 A) and spherical shape particles observed in TEM (Figure 9).

4.4 Challenges in the Precipitation of Quercetin/2-H-P- β Cyclodextrin Under SAS Conditions: Potential Explanations and Limitations

Quercetin/ 2-H-P- β cyclodextrin failed to precipitate under the SAS conditions could be due to multiple possible reasons. Strong host–guest inclusion complexation between quercetin and β -cyclodextrin in ethanol inhibited nucleation: β -CD's hydrophobic cavity sequesters quercetin via robust non-covalent interactions, keeping the complex in solution, whereas an excipient like Pluronic F127 does not form such a tight inclusion and thus co-precipitates more readily – indeed, SAS experiments show pure quercetin crystallizing into needles (Figure 9) while Pluronic F127 induces distinct spherical particles, indicating that quercetin can nucleate and phase-separate when only loosely bound by a polymer (Lévai et al. 2015). In previous SAS experiments ((Adeoye et al. 2018; Liang et al. 2023; Miyake et al. 2000; Wang et al. 2023) the preparation of drug/inclusion complex by 2-HP- β cyclodextrin was run at CO_2 flow rates of 25 mL/min versus the current experiment at 2.5-5 mL/min due to the instrumental limitation to reach such higher flow rates that resulted in greatly reducing antisolvent efficacy thereby insufficient CO_2 influx hence a lower CO_2 molar fraction in the mixture (far below the >95% typically employed) and sluggish solvent extraction, so the solution never experienced the rapid solvent removal and compositional shift required to reach the critical supersaturation threshold. The slower CO_2 diffusion and mixing at 2.5 mL/min prevented the instantaneous oversaturation of quercetin/2- HP- β -CD, and even at 200 bar no precipitation occurred because the system likely entered a single-phase region where the solute remained soluble. As explained in the simulation studies (Antipova, Odintsova, and Petrenko 2024), the beta

cyclodextrin behavior under the supercritical CO₂ was not stable as they observed the formation and breaking up of the complex throughout the process due to the less localization of the ethanol molecules around the inclusion complex and the hydrophobic drug. The flow rate of quercetin/2-HP- β -CD ethanol solution was too low that the optimum amount of fluid density was not achieved as observed also in the Table 3 where no yield obtained. The operating parameters must be optimized well enough to obtain the crystals. According to (De Marco and Reverchon 2008) 2-HP- β -CD poor solubility with supercritical CO₂ promotes the supersaturation and precipitation but 2-HP- β -CD can also increase the drug solubility and that's why the ratio between the ScCO₂ to solvent mixture ratio is a crucial parameter to be optimized.

4.5 Formation of Quercetin 2 HP- β cyclodextrin and Quercetin- PF 127 Nanocrystals using Liquid CO₂ method

Table 4 - Optimization parameters of Quercetin crystals using liquid CO₂ flow rate 2 mL/min for 2 hours and Solvent injection method.

	Formulation	C _{Quercetin} - C _{Polymer} (mM)	Pressure (bars)	% Yield
Liquid CO ₂	Qc- β cd	12 - 12	100	78.3 %
	Qc-PF127	6 - 6	100	76 %
Solvent injection	Qc- β cd	12 - 12	-	83 %
	Qc-PF127	6 - 6	-	66.8 %

In the experiments as shown in the table 4, quercetin/2 HP β -CD and quercetin/Pluronic F127 precipitated successfully under liquid CO₂ conditions (ice-cold 0 °C, 200 bar, CO₂ 2 mL/min) with yields of 78.3% and 76%, respectively. There are various factors that influence the yield, the solubility of Liquid CO₂ firstly with the solvent (ethanol). The solubility interaction are different in between subcritical (liquid) and supercritical states. Liquid CO₂ at ice-cold temperature (0 °C) is highly dense but remains a separate phase from ethanol; it has limited mutual solubility with the organic solvent under these conditions. Thus, when liquid CO₂ is flowed through an ethanol solution, the two phases coexist, and ethanol is extracted gradually via phase boundary partitioning. In contrast, supercritical CO₂ at 40 °C and 200 bar is above the critical point of the mixture with ethanol, meaning CO₂ and ethanol become largely single-phase miscible under these conditions (Boutin and Badens 2009). Under liquid CO₂, the ethanol-rich phase to persist because of the higher density of the liquid CO₂ comparatively to

the Sc-CO₂ limiting the solvent – antisolvent phenomenon. As CO₂ slowly dissolves into the ethanol phase, the solvent power of ethanol is incrementally reduced, concentrating the quercetin/carriers (2 HP β -CD and Pluronic F127) in the shrinking liquid phase. This sets up a progressively increasing supersaturation within the solution until the solubility limit is crossed and nucleation occurs. Essentially, the slow solvent removal allows the solution to reach the critical supersaturation threshold needed for precipitation. Conversely, supercritical CO₂, ethanol is extracted almost instantaneously due to the complete miscibility: Sc-CO₂ diffuses into and expands each droplet of solution extremely rapidly. Under liquid CO₂ conditions, the extraction of ethanol is more gradual, allowing residual solvent to persist in the vessel over the 2-hour run. This slower removal permits the solute to progressively concentrate and ultimately exceed its solubility limit in the diminishing ethanol fraction, thereby promoting supersaturation. In contrast, the aggressive solvent extraction by Sc-CO₂ rapidly depletes ethanol, preventing the necessary build-up of solute concentration to surpass the nucleation threshold and thus precluding precipitation (Wang et al. 2023). Higher pressure generally increases CO₂ density and enhances its antisolvent strength. At 200 bar in both cases the pressure was ample. However, temperature changes CO₂ density at a given pressure. CO₂ density at 40 °C at 200 bars, decreases (and its solvent strength for ethanol decreases) as a result CO₂ density at 40 °C would not hinder ethanol extraction instead the effect is counteracted by the single-phase mixing: even if slightly less dense, Sc-CO₂ at 40 °C still dissolves ethanol readily, but it holds the ethanol in one phase with the solute. At 0 °C, CO₂ density is extremely high (1021 kg / m³) that can dissolve a bit more ethanol at the interface, but it remains a separate phase (so it never carries all the ethanol into one homogeneous phase). The net result: lower temperature promoted precipitation because it kept a concentrated liquid phase of solvent - solute in equilibrium with CO₂, rather than instantaneously diluting it. Temperature also affects kinetics. Lower temperatures generally slow molecular diffusion and particle growth, which can favor nucleation over crystal growth. Lower temperatures promote specific molecular interactions by reducing thermal motion, thereby stabilizing certain complexes. For instance, cyclodextrin complexes tend to exhibit greater stability at lower temperatures, as there is less thermal energy to disrupt their inclusion. Liang et al. observed that “the formation of stable complexes can be facilitated under lower temperature conditions.” (Liang et al. 2023).

In our experiment, under ice-cold conditions, the quercetin- β -CD complex is more likely to remain intact, eventually precipitating once ethanol is sufficiently removed. Conversely, at 40 °C, the increased thermal energy may either weaken drug-carrier interactions or hinder their formation during rapid expansion. Additionally, Pluronic F127 exhibits temperature-dependent behavior, with a defined cloud point and critical micelle temperature in aqueous

systems, though this effect is less pronounced in ethanol. A colder environment may have triggered premature polymer precipitation, potentially co-precipitating quercetin, whereas at 40 °C, both components likely remained dissolved until precipitation occurred at a later stage.

4.6 Dynamic Light Scattering (DLS)

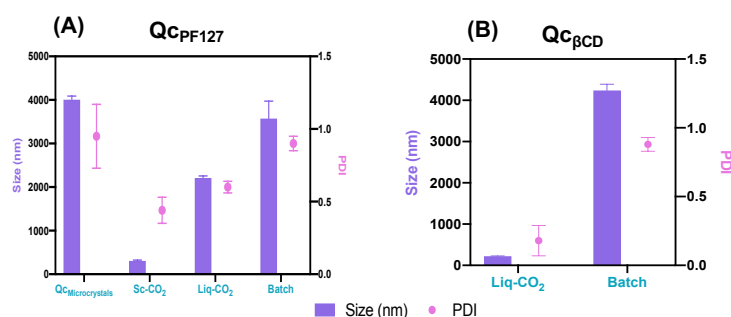


Figure 10 - Particle size distribution and PDI of (A) QcPF127 crystals prepared using Sc-CO₂, Liquid CO₂, and batch methods and (B) Qc 2 HP-β cyclodextrin crystals prepared using Liquid Co₂ and batch method.

The DLS analysis in Figure 10 revealed that the QcPF127 crystals prepared using Sc-CO₂ achieved a unimodal distribution of 300 ± 25.18 nm with a PDI of 0.44 ± 0.09 , whereas the crystals prepared using Liq-CO₂ exhibited markedly larger sizes at 2.2 ± 55.8 μ m with a PDI of 0.6 ± 0.04 , and the batch-prepared sample reached 3.5 ± 404.1 μ m with a PDI of 0.9 ± 0.05 . Additionally, the Qcbcd crystals formulated via Liq-CO₂ demonstrated a particle size of 221.7 ± 8.1 nm and a notably low PDI of 0.18 ± 0.11 , in contrast to the batch-prepared counterpart with a size of 4.2 ± 151.3 μ m and a PDI of 0.88 ± 0.05 . These results underscore the fundamental importance of achieving optimal particle size and homogeneity for oral delivery formulations. In oral drug delivery, maintaining a particle size within the nanometer range of ideally between 200 and 300 nm—is critical to ensure that the particles effectively navigate the gastrointestinal tract and interact with the intestinal villi for efficient absorption. Particles that are too small may be rapidly cleared or inadequately adhere to the absorption sites, while overly large particles risk triggering an immune response or may not readily permeate the mucosal barriers. Moreover, a low PDI (below 0.5) is essential for ensuring a uniform particle distribution. For the supplement-based antioxidant activity, smaller sizes are favored.

Table 5 - Drug loading % of quercetin crystals in Mean $\pm$ SD.

Drug loading % (DL%) of Quercetin				
Sc-CO ₂	Liquid CO ₂		Batch Method	
QCPF127	QCPF127	QCβCD	QCPF127	QCβCD
82 ± 3.7	92 ± 9.2	75 ± 10.4	67 ± 0.6	47 ± 0.8

4.7 Phase Solubility Studies

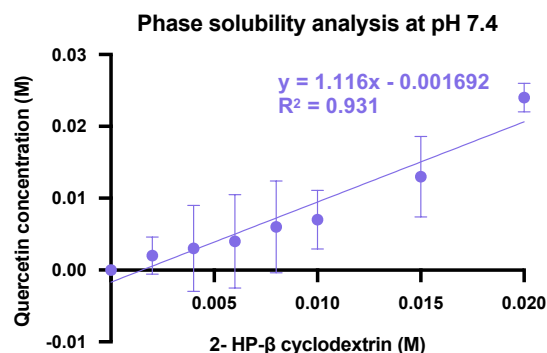


Figure 11 - Phase Solubility curve of Quercetin vs 2- HP-β- cyclodextrin (M).

Drug molecules are readily released from the cyclodextrin complex upon media dilution or through competitive complexation. In principle, one or more drug molecules can complex with a single 2-HP-β-CD molecule, and conversely, one or more 2-HP-β-CD molecules can complex with a single drug molecule. However, the most common stoichiometry—especially in the case of quercetin—is 1:1, where one quercetin molecule complexes with one 2-HP-β-CD molecule, giving rise to an equilibrium constant ($K_{1:1}$). Phase solubility studies elucidate both how much quercetin solubility can be improved via complexation with 2-HP-β-CD and how stable that complex is, as represented by the solubility constant (K_s). According to the Higuchi and Connors classification, various phase solubility diagrams illustrate how total drug solubility changes with rising cyclodextrin concentration: A-type diagrams occur when the drug/CD complex is readily soluble in the aqueous medium—common with water-soluble CD derivatives—while B-type diagrams arise if the complex exhibits limited solubility, often seen with natural cyclodextrins. Subtypes of A-type diagrams include AL (linear), AP (positive deviation), and AN (negative deviation), whereas B-type diagrams can be further categorized as BS (some but limited solubility) or BI (insoluble complex) (Saokham et al. 2018). In the present study (Figure 11), the slope of the experimental equation is 1.116, suggesting that although a slope near or slightly above 1 can imply partial higher-order complex formation (e.g., 1:2), the minimal deviation from 1 indicates that the predominant complex is still 1:1—reflected by an AL-type curve. The intercept (-0.001692), very close to zero, corresponds to quercetin's intrinsic solubility (S_0) in water and confirms its poor aqueous solubility in the absence of cyclodextrin. Moreover, the stability constant (K_s) calculated from the equation is 5.68×10^3 signifies that higher K_s values correlate with increased complex stability. Overall, the linear rise in quercetin solubility with increasing 2-HP-β-CD concentration highlights the substantial solubility enhancement offered by cyclodextrin complexation, and this improved solubility is expected to translate into better bioavailability.

4.8 Fourier Transform- Infrared Spectroscopy (FTIR)

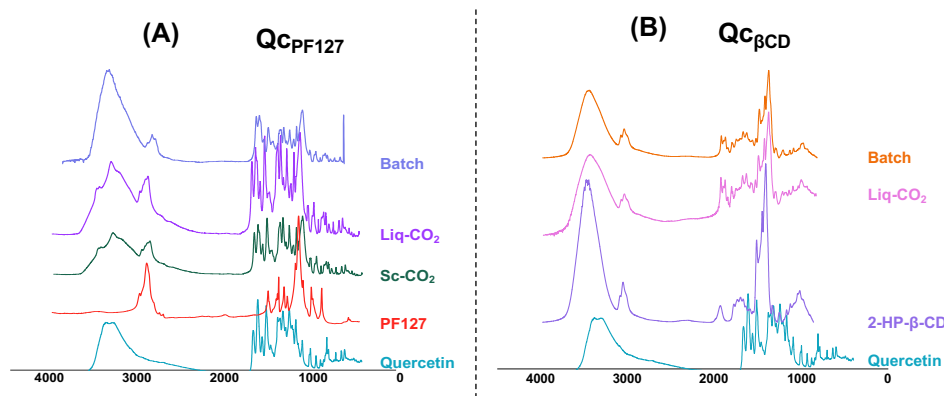


Figure 12 - FTIR spectra of (A) pure quercetin, Pluronic F-127 (PF127), and QcPF127 formulations prepared using Supercritical CO₂ (Sc-CO₂), Liquid CO₂ (Liq-CO₂), and the batch method; and (B) 2-hydroxypropyl-β-cyclodextrin (QcβCD) formulations obtained via Liquid CO₂ (Liq-CO₂) and the batch method.

4.8.1 Qc PF127 crystal formulations

The broad O–H stretching band observed between 3000 and 3600 cm⁻¹ is primarily attributed to its phenolic hydroxyl groups, which are highly capable of engaging in hydrogen bonding. This broad band not only reflects the presence of these functional groups but also indicates the extent of their involvement in intermolecular interactions. Quercetin exhibits a prominent C=O stretching vibration near 1650 cm⁻¹, which originates from the conjugated carbonyl group present in its flavonoid structure, this peak is critical for assessing any alterations in the electronic environment of quercetin upon complex formation or encapsulation. Furthermore, the aromatic ring vibrations appearing in the 1500–1600 cm⁻¹ region serve as clear markers of the flavonoid aromatic system, providing further confirmation of its structural integrity.

Pluronic F127 display most notably the C–O–C stretching vibrations that appear around 1100–1150 cm⁻¹, a reflection of the ether linkages within its polyethylene oxide (PEO) and polypropylene oxide (PPO) blocks. There is also a weak –OH terminal group signal near 3400 cm⁻¹, although this is often obscured by the more intense, broad O–H stretching band of quercetin when the two components are present in a crystal.

The FTIR spectra of QcPF127 crystals, prepared via distinct solvent environments—including the ethanol solvent injection (batch method), the Supercritical CO₂ (Sc-CO₂) and liquid CO₂ (Liq-CO₂) as solvents and antisolvents—exhibit marked differences in molecular interactions, solvation effects, and crystallinity that are vividly reflected in the spectral shifts of key functional groups. In the batch-prepared samples, where ethanol predominates as the solvent, quercetin retains its pronounced crystallinity, as evidenced by the sharp, well-defined O–H stretching bands in the 3300–3600 cm⁻¹ region and the distinct C=O stretching peak at approximately 1650 cm⁻¹, indicative of minimal hydrogen bonding with Pluronic F127; furthermore, the characteristic ether (–C–O–C–) peaks of Pluronic F127, observed between 1100 and 1150

cm^{-1} , remain largely unaltered, underscoring the limited polymer–drug interaction as also observed in lower DL%.

In contrast, the Liq- CO_2 as an antisolvent—where quercetin and Pluronic F127 are initially dissolved in ethanol—elicits moderate spectral shifts; the O–H band broadens and undergoes a slight red-shift, suggesting partial hydrogen bonding and an attendant reduction in crystallinity, while a modest diminution in the C=O peak intensity at around 1650 cm^{-1} , accompanied by minor shifts, signifies partial encapsulation within the polymer matrix and improved molecular dispersion.

Sc- CO_2 method manifests the most significant alterations: the O–H stretching band, originally centered near 3300 cm^{-1} , broadens markedly and shifts further to lower wavenumbers, evidencing robust hydrogen bonding between quercetin and the PEO segments of Pluronic F127; concurrently, the C=O stretching peak at approximately 1650 cm^{-1} exhibits a pronounced decrease in intensity and slight shifting, indicative of substantial amorphization and molecular-level complexation, while the ether vibrations of Pluronic F127 in the $1100\text{--}1150\text{ cm}^{-1}$ region display noticeable broadening, reflecting polymer conformational changes thus also providing the higher degree of drug loading.

4.8.2 Qc β CD crystal formulations

Pure 2HP- β -CD exhibits a similarly broad O–H band in the $3000\text{--}3500\text{ cm}^{-1}$ region due to the numerous hydroxyl moieties in its ring structure; one also observes C–O stretching vibrations and glycosidic ring modes around $1100\text{--}1000\text{ cm}^{-1}$, which are indicative of pyranose rings and ether linkages within the cyclodextrin framework while in the regions $2800\text{--}3000\text{ cm}^{-1}$ appears the vibrations of -CH and - CH_2 groups. When quercetin is incorporated into 2HP- β -CD via the batch method, the FTIR profile reveals a partial overlap of quercetin O–H stretch ($3000\text{--}3600\text{ cm}^{-1}$) with that of 2HP- β -CD, signifying moderate hydrogen bonding interactions and partial encapsulation. Moreover, the C=O peak of quercetin near 1650 cm^{-1} may undergo a slight shift or change in intensity, hinting at the onset of complexation while preserving some degree of the pure flavonoid crystallinity. Conversely, the crystals prepared through the Liq- CO_2 method exhibits a more pronounced broadening of the O–H region ($3000\text{--}3600\text{ cm}^{-1}$) and a more substantial attenuation or shifting of the quercetin carbonyl peak around 1650 cm^{-1} , suggesting a greater degree of molecular entrapment and stronger hydrogen bonding within the cyclodextrin cavity. Additionally, the CH and CH_2 bands in the $2800\text{--}3000\text{ cm}^{-1}$ region of the crystals prepared using both methods display enhanced clarity, indicating that the aliphatic environment of 2HP- β -CD remains intact while also participating in the formation of the inclusion complex. These spectral modifications mentioned above implies that Liq- CO_2 processing enhances drug–carrier interactions and reduces the crystallinity of quercetin more

effectively than the batch approach, culminating in a more cohesive complex that is consistent with improved loading (75 %) and dispersion of quercetin within the 2HP- β -CD matrix.

4.9 Differential Scanning Microscopy (DSC)

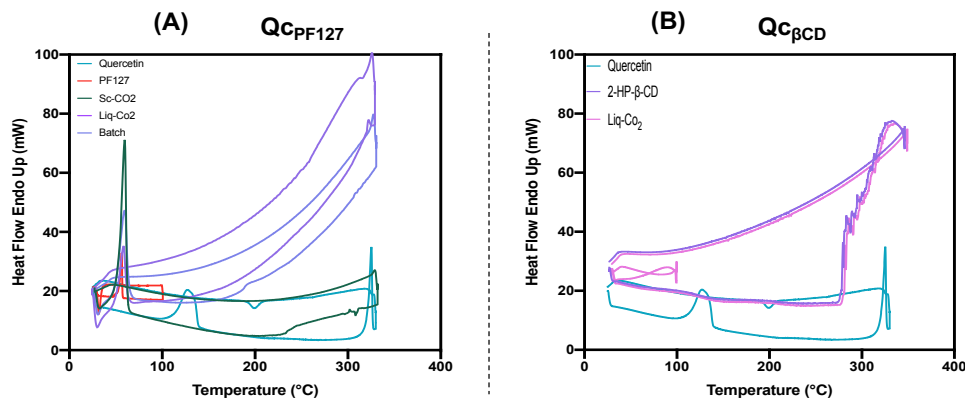


Figure 13 - DSC thermogram of (A) pure quercetin, Pluronic F-127 (PF127), and QcPF127 formulations prepared using Supercritical CO₂ (Sc-CO₂), Liquid CO₂ (Liq-CO₂), and the batch method; and (B) 2-hydroxypropyl- β -cyclodextrin (Qc β CD) formulations obtained via Liquid CO₂ (Liq-CO₂).

4.9.1 QcPF127 crystal thermogram A

Quercetin exhibits two melting events: one occurring between 110 and 136.4 °C, peaking at 127.2 °C with a heat of fusion of 230 J/g suggesting the loss of bound water and another from 323.4 to 328.2 °C, peaking at 326.9 °C with a heat of fusion of 115 J/g indicates the distinct crystalline arrangement representing the quercetin melting point. In contrast, pure Pluronic F-127 shows a more uniform thermal transition with a melting onset between 53.2 and 56.4 °C and a main peak at 55.5 °C during the first cycle, accompanied by a heat of fusion of 126.5 J/g. The second cycle exhibits a slight shift in the onset (51.1–55.4 °C) and a marginal reduction in the main peak temperature (54.6 °C) with a heat of fusion of 121 J/g, indicating consistent yet subtly altered thermal behavior upon repeated heating. Meanwhile, in the crystal sample the first quercetin peak was not visible indicating the water was removed by CO₂ itself. The observation that the heat of fusion of the crystalline modifications in the crystals is lower than that of the pristine crystalline material suggests a reduction in the degree of crystallinity or a transition towards a less ordered state. Furthermore, the Qc/PF127 formulation prepared by Sc-CO₂ method demonstrates an endothermic peak with a heat flow in the range of 20–30 mW, which is significantly subdued compared to the Liq-CO₂ and batch-prepared samples that reached up to 100 mW before receding to about 20 mW. This reduced endothermic response in the Sc-CO₂ sample is indicative of a more efficient molecular dispersion and a higher degree of amorphization of quercetin within the PF127 matrix, ultimately requiring less energy for the phase transition and thereby potentially enhancing the solubility and bioavailability of the drug.

4.9.2 Qc β CD crystal thermogram B

The DSC thermogram for 2HP- β -CD reveals a sharply defined thermal transition that commences with an onset at approximately 110 °C, culminating in a distinct endothermic peak at 167 °C. This peak is indicative of the crystalline melting process of the cyclodextrin, characterized by an efficient heat transfer over a narrow temperature interval and a measured heat of fusion of 84 J/g. During the cooling cycle, the sample exhibits a complete degradation beginning at around 200 °C rather than undergoing recrystallization, suggesting that the thermal stability of the material is compromised at elevated temperatures, leading to irreversible decomposition.

In the case of the complex represented by the pink curve, the initial heating-cooling cycle mirrors the cyclodextrin's inherent behavior, displaying the characteristic endothermic peak associated with its melting process. Presence of no quercetin peak indicate the interaction and complexation with the cyclodextrin. However, upon execution of a second cycle, the complex demonstrates an identical thermal profile and degradation pattern to that of the pure 2HP- β -CD.

4.10 X-ray diffraction (XRD)

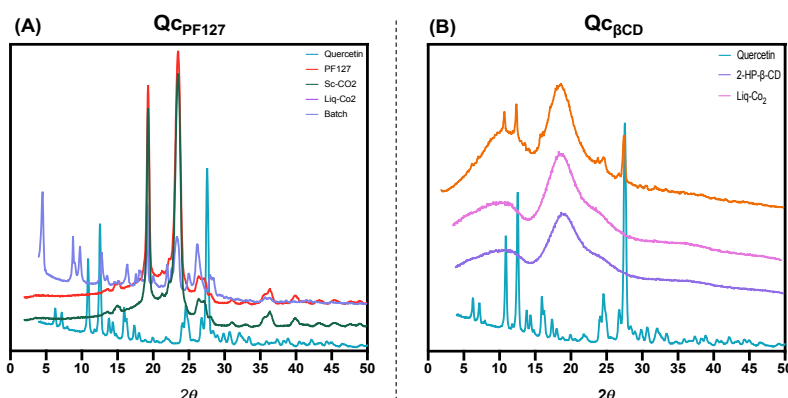


Figure 14 - XRD diffractogram (A) pure quercetin, Pluronic F-127 (PF127), and QcPF127 formulations prepared using Supercritical CO₂ (Sc-CO₂), Liquid CO₂ (Liq-CO₂), and the batch method; and (B) 2-hydroxypropyl- β -cyclodextrin (Qc β CD) formulations obtained via Liquid CO₂ (Liq-CO₂) and batch methods.

The diffractogram of quercetin corroborates literature data (57), revealing its high crystallinity with distinct peaks at 6.28°, 7.18°, 10.88°, 12.54°, 13.79°, 15.94°, 17.35°, 18.02°, 18.85°, 21.81°, 24.59°, 26.76°, 27.56°, 29.86°, 33.42°, 35.86°, 37.37°, and 46.54°. In contrast, Pluronic F127 (PF127) exhibits its typical sharp peaks at 15.28°, 19.36°, 23.56°, 31.12°, 36.40° in agreement with the findings of (Fraile et al. 2014). Complexes prepared using Sc-CO₂ and Liq-CO₂ displayed predominantly the characteristic peaks of PF127 with quercetin peaks scarcely observed, implying that quercetin is well incorporated within an amorphous matrix. Conversely, the batch-prepared complex demonstrated a distinct diffraction pattern, with the prominent presence of quercetin peaks alongside those of PF127, suggesting limited

molecular interaction between the two components. This phenomenon is attributed to the strong binding of quercetin with PF127, an inherently amorphous molecule, leading to the formation of an amorphous complex.

In addition, the XRD analysis of 2 HP- β -CD, as reported by (Wang et al. 2023), showed characteristic peaks at 10.37° and 19.18° . In the batch-prepared complex, the presence of the quercetin peak alongside a broad cyclodextrin peak at 12.55° , 18.69° and 27.63° indicates minimal interaction between them. However, in the Liq- CO_2 prepared complex, the diffractogram resembled that of cyclodextrin's amorphous nature, suggesting robust intermolecular interactions.

Amorphous materials, owing to their lack of long-range molecular order, typically exhibit higher solubility and faster dissolution rates compared to their crystalline counterparts. This enhanced solubility is critical for oral delivery systems, as it facilitates rapid and efficient drug release and absorption in the gastrointestinal tract. Furthermore, the increased molecular mobility in amorphous formulations can lead to improved bioavailability, particularly for poorly soluble compounds like quercetin.

4.11 In vitro drug release

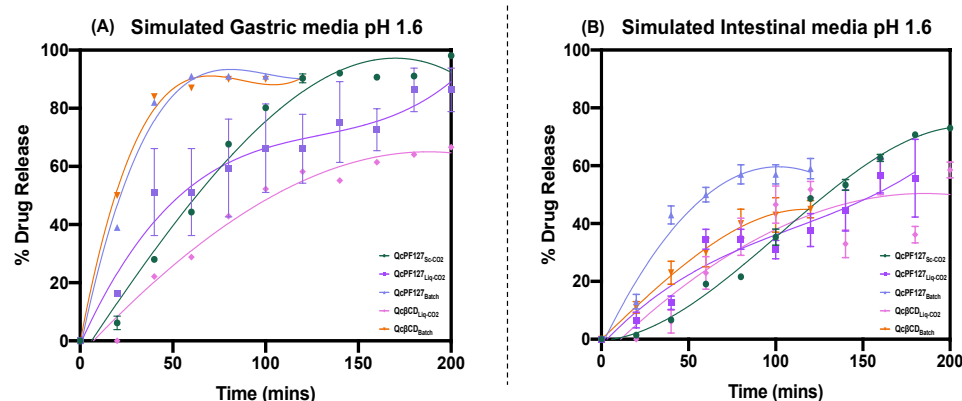


Figure 15 - In vitro drug release (A) Simulated Gastric media pH 1.6 and (B) Simulated Intestinal media pH 6.8 of QcPF127 and QcβCD formulations.

4.11.1 QcPF127 crystal formulations

In vitro drug release studies conducted at gastric pH 1.6 Figure 15a, demonstrated that the QcPF127 formulation prepared via supercritical CO_2 (Sc-CO_2) achieved $98.10 \pm 0.9\%$ release by 200 minutes, surpassing both the liquid CO_2 formulation ($86.34 \pm 7.4\%$) and the batch formulation ($91 \pm 2\%$). Notably, the batch-prepared sample exhibited a rapid initial release, reaching approximately 91% within 80 minutes, after which it plateaued and remained constant up to 200 minutes. Structural characterizations suggest that this rapid release may stem from weaker drug-polymer interactions in the batch formulation, whereas the Sc-CO_2 and liquid CO_2 formulations demonstrate stronger interactions that foster a more sustained release. Specifically, the Sc-CO_2 formulation reached 90% release by 120 minutes and

progressed to 98% by 200 minutes, while the liquid CO₂ formulation increased from 66% at 120 minutes to 86% at 200 minutes, reflecting a consistent, albeit slower, release pattern. When evaluated in an intestinal medium with pH 6.8 (Figure 15 b) shows both the Sc-CO₂ and liquid CO₂ formulations controlled and gradual release profiles, achieving $73 \pm 0.9\%$ and $56.48 \pm 2.7\%$ release, respectively, at 200 minutes. In contrast, the batch formulation quickly reached $59 \pm 4\%$ by 120 minutes but did not exceed this value thereafter. Literature suggests that quercetin undergoes partial degradation in alkaline conditions, which likely explains the limited further release observed in the batch formulation once the pH reached intestinal levels. Quercetin, inherently limited by its low aqueous solubility, exhibits modest solubility improvements in acidic environments but remains prone to degradation in alkaline conditions, which constrains its overall release in intestinal media. PF127 facilitates the formation of micellar structures that enhance quercetin dispersion, thereby augmenting its solubility in the gastric phase and ensuring a more controlled release in the intestine; however, it cannot entirely avert degradation induced by elevated pH. Consequently, the Sc-CO₂ method achieves nearly complete release under both acidic and moderately alkaline conditions, while the liquid CO₂ technique provides a moderately sustained release profile, and the batch process, despite its rapid initial release it ultimately exhibits reduced intestinal release due to weaker drug–polymer interactions and the pH-sensitive degradation of quercetin.

4.11.2 QcβCD crystal formulations

In vitro release studies at gastric pH 1.6, as illustrated in Figure 15(a), demonstrated that the Liq-CO₂ prepared formulation of QcβCD exhibited a gradual release reaching $66.66 \pm 4.62\%$ up to 200 minutes, whereas the batch-prepared crystals achieved a rapid release of $90 \pm 1\%$ by 120 minutes, indicative of an initial burst effect likely arising from weaker drug–polymer interactions. Conversely, at intestinal pH 6.8, the Liq-CO₂ prepared crystals maintained a release of $58.52 \pm 2.76\%$ up to 200 minutes compared to $45 \pm 3\%$ for the batch-prepared formulation at 120 minutes. This disparity can be attributed to the enhanced molecular cohesion and superior stability inherent in the Liq-CO₂ method, which, while moderating the initial release rate, afford a more sustained and controlled drug diffusion in neutral media.

2HP-β-CD, with its hydrophobic cavity, forms inclusion complexes with quercetin that can enhance solubility yet often result in a more stable, and thus less readily dissociable, association, potentially retarding the drug release. In contrast, Pluronic F127, an amphiphilic triblock copolymer, self-assembles into micellar structures that encapsulate quercetin within a hydrophobic core, promoting not only improved aqueous dispersion but also a more dynamic, controlled release profile in both acidic and neutral environments. Thus, the micellar loading of the quercetin as observed in the table 5 presents 90-80% facilitates a more efficient release of quercetin, as the drug is gradually liberated from the micelles into the surrounding medium,

whereas the stronger, less labile inclusion complex formed with beta-cyclodextrin may impede rapid drug liberation despite enhancing initial solubility.

4.12 Antioxidant analysis

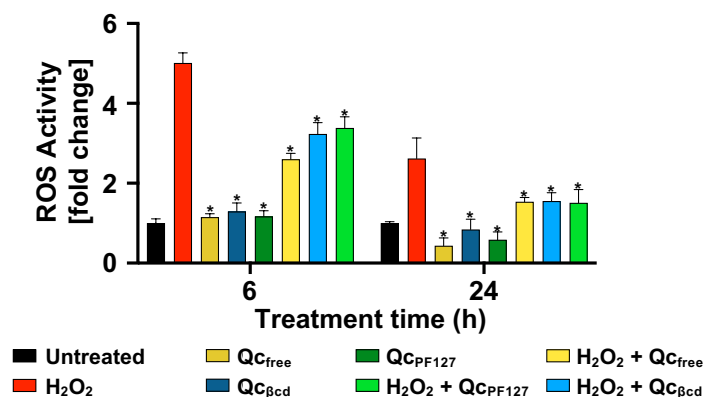


Figure 16 - Intracellular ROS levels in MRC-5 cells treated for 6 or 24 hours under different conditions. Cells were either left untreated, exposed to 250 μ M H₂O₂ (oxidative control), or treated with 50 μ M quercetin in free form (Qc_{free}), F127-encapsulated quercetin (Qc_{PF127}), or quercetin in β -cyclodextrin (Qc_{βcd}), alone or in combination with H₂O₂. Data are presented as mean $\pm$ SD (n=9). Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test, comparing each treatment with H₂O₂. Asterisks indicate significant differences relative to the H₂O₂ control group.

All ROS measurements were normalized to the untreated control (baseline 1.00). The ROS generation on MRC-5 achieved treating the cells with 250 μ M H₂O₂ reached 5.01 ± 0.25 at 6 hours and decreasing to 2.62 ± 0.51 at 24 hours. In contrast, treatment with 50 μ M Qc_{free} maintained ROS levels near baseline, at 1.15 ± 0.08 after 6 hours and further reducing to 0.44 ± 0.19 at 24 hours. Similarly, Qc_{βcd} exhibited values of 1.29 ± 0.21 at 6 hours and 0.84 ± 0.26 at 24 hours, while Qc_{PF127} showed levels of 1.17 ± 0.14 and 0.58 ± 0.20 at 6 and 24 hours, respectively. Notably, when H₂O₂ was co-administered with the quercetin formulations, ROS levels were significantly mitigated: the combination with Qc_{free} yielded 2.60 ± 0.15 (48.1% reduction) at 6 hours and 1.54 ± 0.11 (41.2% reduction) at 24 hours; with Qc_{βcd}, the values were 3.23 ± 0.28 (35.5% reduction) at 6 hours and 1.56 ± 0.21 (40.5% reduction) at 24 hours; and with Qc_{PF127}, the measurements were 3.38 ± 0.28 (32.5% reduction) at 6 hours and 1.51 ± 0.33 (42.4% reduction) at 24 hours. The aglycone part of quercetin is a powerful direct radical-scavenger : the catechol B-ring (3',4'-dihydroxy) and the 3-hydroxyl on the C-ring donate hydrogen atoms/electrons to neutralize H₂O₂-derived hydroxyl and superoxide radicals, while its π -conjugated backbone delocalises the resulting flavonoxyl radical and prevents chain propagation. Once inside the cell quercetin activates the Nrf2/ARE pathway, up-regulating endogenous defences such as HO-1, SOD, and catalase; this indirect component emerges more slowly and dominates after several hours. The nanocrystal formulations do not alter quercetin chemistry but boost its bioavailability at the cellular interface and acts as a slow release reservoirs. These data clearly demonstrate that the formulations effectively attenuates H₂O₂-induced oxidative stress in MRC-5 cells across both time points.

5. Conclusions

Overall, this work underscores the profound influence of processing methods on quercetin's physicochemical properties, encapsulation efficiency, and antioxidant efficacy. Supercritical CO₂ (Sc-CO₂) processing proved particularly effective at enhancing molecular dispersion and drug loading, especially when combined with Pluronic F127, resulting in near-complete drug release under acidic conditions and a sustained, controlled release in more alkaline media. This method also demonstrated robust mitigation of H₂O₂-induced ROS generation in MRC-5 cells, highlighting the potential of these formulations for effective quercetin delivery to counteract oxidative stress-related conditions. Liquid CO₂ processing, by contrast, offered a gentler solvent extraction mechanism that favored a gradual build-up of supersaturation, thereby yielding moderately high drug loading and controlled release profiles when quercetin was complexed with either Pluronic F127 or β -cyclodextrin. In comparison, the batch (solvent injection) method produced crystals with relatively higher crystallinity, as evidenced by sharper XRD peaks and distinct thermal transitions but resulted in a rapid initial release in gastric conditions that plateaued under intestinal pH, likely due to weaker polymer–drug interactions and the pH-sensitive degradation of quercetin. Despite these differences, all formulations significantly mitigated oxidative stress, emphasizing quercetin's intrinsic antioxidant potential. Moreover, the comparative analyses using FTIR, DSC, and XRD confirmed that the degree of molecular interaction and crystallinity directly correlates with the observed drug release and bioavailability profiles. Pluronic F127, by facilitating micellar formation, enhances quercetin dispersion and promotes a dynamic and controlled release mechanism, while β -cyclodextrin, through its hydrophobic cavity, forms stable inclusion complexes that, although improving initial solubility, may sometimes retard complete drug liberation. Looking ahead, future research should aim to refine CO₂-based processing parameters—including flow rate, pressure, and solvent ratios—to optimize particle size, drug loading, and release kinetics. Scaling up these techniques for industrial production remains a critical challenge that warrants investigation, alongside *in vivo* studies to confirm the enhanced bioavailability and therapeutic efficacy observed *in vitro*. Additionally, exploring alternative biocompatible polymers and excipients, as well as integrating advanced characterization methods, could further enhance formulation performance. Such endeavors will be instrumental in tailoring quercetin-based delivery systems to target specific pathologies associated with oxidative stress, ultimately advancing the field of functional bioactive compounds in pharmaceutical applications.

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Disclaimer

This manuscript has undergone grammar and syntax correction using ChatGPT, an AI language model developed by OpenAI. While every effort has been made to improve the clarity and accuracy of the language in this document, the authors acknowledge that the final content and scientific interpretations remain the sole responsibility of the authors and their collaborators. ChatGPT has been used exclusively to enhance the manuscript's readability and expression, and not to generate any type of data.

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Chapter 2

Microfluidic-Assisted Formulation and Characterization of Quercetin-Loaded Liposomes for Enhanced Oral Delivery

1. Introduction

The development of new drug molecules is an endeavor fraught with exorbitant costs, extensive timelines, and a multitude of regulatory hurdles. In the relentless pursuit of optimizing therapeutic efficacy while minimizing adverse effects, the refinement of existing pharmacological agents has emerged as a pragmatic alternative. The intricate interplay between drug pharmacokinetics and pharmacodynamics necessitates precise modulation of drug bioavailability, biodistribution, and metabolic stability to achieve optimal therapeutic indices. Conventional drug administration, though the cornerstone of pharmacotherapy, is often hampered by systemic inefficiencies, such as erratic absorption, premature degradation, suboptimal biodistribution, and an inherent lack of specificity, which collectively compromise clinical outcomes (Lesko and Schmidt 2012)

Drug delivery systems (DDS) have materialized as an indispensable frontier in modern pharmaceutical sciences, revolutionizing the paradigm of therapeutic administration by conferring heightened control over drug release kinetics, spatial targeting, and systemic exposure (Tiwari et al. 2012). These sophisticated carriers, often formulated using biocompatible lipids and polymers, function as reservoirs that meticulously modulate drug dispersal, ensuring a sustained and localized therapeutic effect while circumventing the pitfalls of conventional modalities. By leveraging principles of nanotechnology, biomaterials science, and pharmaceutical engineering, DDS not only mitigate deleterious off-target effects but also potentiate therapeutic efficacy through precise localization at the intended site of action (Al-Jamal and Kostarelos 2011)

The evolution of DDS is underscored by their ability to shield pharmacological agents from enzymatic degradation and rapid systemic clearance, thereby prolonging circulatory half-life and amplifying drug retention within pathological tissues. This strategic modulation of drug fate within the biological milieu facilitates lower dosage regimens, effectively minimizing systemic toxicity and enhancing patient compliance (Manzari et al. 2021). Encapsulation within particulate carriers, such as liposomes, micelles, dendrimers, or polymeric nanoparticles, serves to augment solubility, permeability, and controlled dissolution, further refining the pharmacological profile of administered therapeutics (Anselmo and Mitragotri 2014). In oncology, for instance, DDS have enabled targeted chemotherapy, wherein cytotoxic agents are preferentially delivered to tumor microenvironments via ligand-receptor interactions or enhanced permeability and retention (EPR) effects, thereby sparing healthy tissues from collateral damage (Maeda et al. 2000). Similarly, in chronic inflammatory disorders and neurodegenerative diseases, DDS have been instrumental in crossing physiological barriers, such as the blood-brain barrier (BBB), to facilitate precise drug accumulation in affected regions (Bors and Erdő 2019). Despite their unequivocal promise, the widespread integration

of DDS into mainstream clinical practice is met with formidable challenges. The inherent complexity of DDS formulations often necessitates extensive optimization to ensure reproducibility, scalability, and long-term stability. Moreover, intellectual property (IP) considerations surrounding carrier technology, drug-carrier conjugation, and formulation-specific attributes impose legal and commercial constraints that may impede seamless translation from laboratory research to commercial viability. Cost implications associated with large-scale production and regulatory approval further delineate the barriers to widespread adoption, necessitating a delicate balance between technological innovation and economic feasibility (Hua et al. 2018; Peters et al. 1995; Smith et al. 2002).

1.1 The Overlapping Nature of Drug delivery systems

The structural complexity of DDS necessitates a nuanced approach to their classification, encompassing dosage form, carrier technology, and physiological route, each contributing to a holistic understanding of drug conveyance.

From a **dosage form-based perspective**, DDS are categorized according to conventional pharmaceutical formulations such as tablets, capsules, and injectables, which predominantly define the mode of drug presentation to the patient. This classification underscores critical parameters, including stability, dissolution kinetics, and release profiles, which collectively influence drug bioavailability and therapeutic efficacy. The fundamental interplay between these attributes dictates the pharmacokinetic trajectory of active pharmaceutical ingredients (APIs) and their subsequent biological impact (Vargason, Anselmo, and Mitragotri 2021)

The **carrier-based classification**, in contrast, encapsulates the intricate realm of advanced formulation strategies aimed at augmenting drug solubility, bioavailability, and site-specific delivery. Lipid-based carriers, including liposomes and solid lipid nanoparticles, polymeric nanoparticles, dendrimers, and hydrogels, exemplify this paradigm by enabling controlled, sustained, and targeted release. Such carriers not only extend the systemic half-life of encapsulated drugs but also shield them from premature metabolic degradation, ensuring optimal drug localization at the intended therapeutic site (Wilczewska et al. 2012)

A **route-based classification**, meanwhile, delineates DDS based on the physiological pathways through which drugs traverse within the human body, encompassing oral, transdermal, pulmonary, intravenous, and intranasal administration, among others. Each route presents distinct anatomical and physiological barriers, necessitating tailored DDS designs to optimize drug absorption, biodistribution, and therapeutic performance. The complexity of biological barriers, such as enzymatic degradation in the gastrointestinal tract or the restrictive nature of the blood-brain barrier (BBB), underscores the critical role of route-specific DDS strategies in enhancing drug efficacy and minimizing systemic toxicity (Jain 2020)

The convergence of these three classification paradigms highlights the multifaceted nature of drug delivery. Among the diverse drug administration routes, the oral route remains the most preferred due to its non-invasiveness, patient convenience, and cost-effectiveness. However, oral drug delivery faces significant pharmacokinetic and physiological challenges, including first-pass metabolism, enzymatic degradation in the gastrointestinal (GI) tract, and poor solubility of many bioactive compounds. These barriers necessitate the development of sophisticated drug carriers that can enhance solubility, protect against degradation, and facilitate efficient absorption across intestinal membranes (Chang and Yeh 2012).

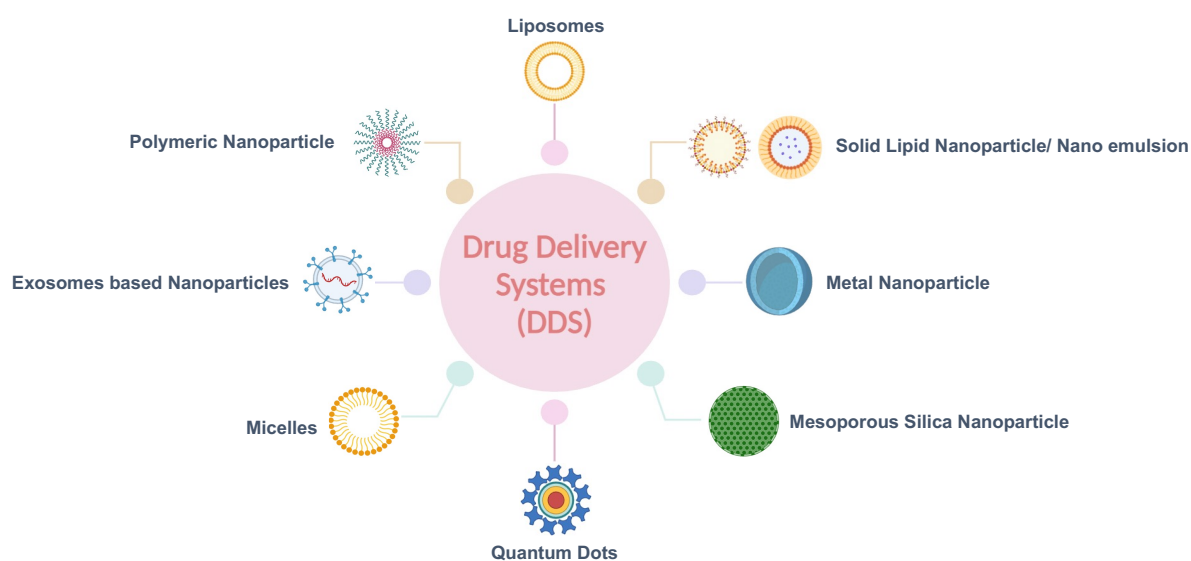


Figure 1 - Carrier based Drug Delivery Systems (Chang and Yeh 2012).

1.2 Advantages of liposomal carrier

Among the myriad of available drug delivery systems, liposomes stand out as a preferred choice due to their unparalleled biocompatibility, tunable physicochemical properties, and ability to encapsulate both hydrophilic and hydrophobic drug molecules (Nsairat et al. 2022). Unlike polymeric nanoparticles or dendrimers, which may exhibit cytotoxicity or elicit undesirable immune responses, liposomes are composed of naturally occurring phospholipids, reducing the likelihood of adverse biological interactions (Yusuf et al. 2023). Additionally, their structural similarity to biological membranes facilitates cellular uptake via endocytosis and fusion, enabling efficient intracellular drug delivery (Li et al. 2011). In comparison to other particulate carriers, such as solid lipid nanoparticles (SLNs) or polymeric micelles, liposomes offer superior flexibility in modulating drug release profiles, targeting efficiency, and surface modifications. The ability to functionalize liposomal surfaces with ligands, antibodies, or peptides allows for active targeting of diseased tissues, a feature less adaptable in rigid polymeric systems (Dattani et al. 2023). Furthermore, their aqueous core provides a stable environment for the encapsulation of biomolecules, including proteins,

peptides, and nucleic acids, preserving their structural integrity and bioactivity (Joshi et al. 2016). Another compelling advantage of liposomes over other carriers lies in their extensive clinical validation and regulatory approval for therapeutic use. Liposomal formulations have been successfully employed in numerous FDA-approved drug products, including Doxil® and Ambisome®, underscoring their safety, efficacy, and translational potential as presented in the Table 1 (Bulbake et al. 2017).

This intrinsic propensity towards bilayer formation in aqueous media is governed by fundamental molecular interactions, including van der Waals forces that maintain lipid tail cohesion and hydrogen bonding that stabilizes interfacial alignment (Euliss et al. 2006). The self-assembly of phospholipids into bilayered vesicles creates a dynamic and protective microenvironment capable of encapsulating hydrophilic and lipophilic molecules alike. The integrity of the lipid bilayer, held together by hydrophobic interactions and electrostatic forces, serves as a semi-permeable barrier that dictates drug retention, controlled release, and protection from premature degradation (Papahadjopoulos and Kimelberg 1974). Beyond structural stability, the behaviour of liposomal membranes is intricately modulated by external parameters such as ionic strength, temperature, and pH fluctuations, allowing the tailoring of liposomal formulations to specific biomedical applications (Wang et al. 2023). These biomimetic vesicles possess an inherent ability to respond to environmental stimuli, a characteristic that has been extensively exploited in designing responsive drug delivery platforms. Lipid-based vesicles, constructed with thermosensitive or pH-sensitive lipids, can undergo destabilization in response to localized physiological cues, thereby ensuring the site-specific release of therapeutic payloads. Such properties have positioned liposomes at the forefront of nanomedicine, particularly in oncology, where targeted drug delivery is paramount in minimizing systemic toxicity while enhancing therapeutic efficacy (Paramshetti et al. 2024). Liposome morphology and phase behaviour are pivotal factors influencing their interaction with biological membranes, endocytic pathways, and intracellular trafficking mechanisms. The fine balance between rigidity and fluidity within the bilayer determines vesicular stability, fusion propensity, and permeability, attributes that are dictated by the ratio of phospholipids to cholesterol and the presence of unsaturated acyl chains (Binder, Barragan, and Menger 2003). The selection of FDA-approved phospholipids and cholesterol for liposomal formulations ensures their biocompatibility and regulatory compliance. Among the widely accepted phospholipids, hydrogenated soy phosphatidylcholine (HSPC), distearoylphosphatidylcholine (DSPC), and egg phosphatidylcholine (EPC) are commonly used for intravenous and oral liposomal formulations due to their stability and ability to form bilayers with optimal fluidity (Drescher and van Hoogevest 2020). Cholesterol, an essential component in many liposomal formulations, is also FDA-approved and plays a crucial role in

enhancing membrane rigidity, reducing permeability, and prolonging systemic circulation by stabilizing the lipid bilayer (Briuglia et al. 2015).

For oral liposomal supplement formulations, plant-based lipids and phytosterols have gained attention for their role in supplement delivery, particularly for the encapsulation of flavonoids. These natural lipid sources offer enhanced biocompatibility, improved stability, and targeted bioavailability, making them ideal for oral nutraceutical applications (Singh et al. 2023). Phospholipids derived from soy lecithin and sunflower lecithin, as well as phytosterols such as beta-sitosterol and stigmasterol, serve as viable alternatives to cholesterol for membrane stability meanwhile providing the cost effectiveness (Wang, Acevedo, and Marangoni 2017). The phospholipid and phytosterol ensures improved absorption and prolonged systemic circulation of encapsulated bioactive compounds. The incorporation of these plant-derived components in liposomal supplements has shown potential in increasing the bioavailability of flavonoids, which often suffer from poor solubility and rapid metabolism in conventional formulations (Kyriakoudi et al. 2021). In the table 2 describes the oral liposomal supplements in the market and widely used worldwide (Altrient 2025; Core Med Science 2025; Indena 2025; QuickSilver Scientific 2025).

Table 1 - FDA approved oral and parenteral liposomal formulations.

Route of Administration	Formulation	Type of lipids	Active component	Primary Use
Oral (Supplement)	Lypospheric® Altrient®	Soy Lecithin, Sunflower Lecithin	Vitamin C	Building immunity, antioxidant
	Core Med Science® Quicksilver Scientific®	Soy Lecithin, Sunflower Lecithin	Glutathione	Antioxidant, detoxification
	Indena Phytosome™ - Quercefit	Soy Lecithin	Quercetin	Antioxidant
	Meriva®	Soy Lecithin	Curcumin	Anti-inflammatory, Antioxidant
Parenteral (i.v)	Doxil®	HSPC: Chol: mPEG-DSPE	Doxorubicin	Ovarian cancer, Multiple Myeloma
	Onivyde®	DSPC: Chol: mPEG-DSPE	Irinotecan	Metastatic pancreatic cancer
	AmBisome	HSPC: Chol: DSPG	Amphotericin B	Systemic fungal infections
	Arikayce	DPPC: Chol	Amikacin	Antibacterial Mycobacterium avium complex (lung disease)

1.3 Conventional technologies for Liposomes Preparation

1.3.1 Thin-film hydration technique

Thin film dispersion method, also known as the Bangham method, remains one of the most widely employed techniques for liposome synthesis. In this process, phospholipids, often in combination with lipophilic drugs, are dissolved in an organic solvent such as chloroform, which is subsequently evaporated under reduced pressure to form a thin lipid film. Upon hydration with an aqueous buffer containing hydrophilic drugs, the lipid film spontaneously reorganizes into multilamellar vesicles. However, to achieve uniformity and reduce polydispersity, further processing through ultrasonication or membrane extrusion is often necessary, refining liposomal dimensions and enhancing their stability (Zhang 2017).

1.3.2 Reverse-phase evaporation method

This technique offers an alternative strategy based on the formation of inverted micelles, wherein phospholipids are dissolved in an organic solvent such as chloroform or ether, followed by the addition of an aqueous drug solution under ultrasonic treatment to form a water-in-oil emulsion. Subsequent removal of the organic phase under vacuum induces vesicle formation, predominantly yielding large unilamellar liposomes. Despite its efficiency, this method presents challenges related to residual solvent contamination, which can be mitigated by employing supercritical carbon dioxide instead of conventional organic solvents, thereby improving the biocompatibility of the final formulation (Szoka Jr and Papahadjopoulos 1978).

1.3.3 Chemical gradient-based methods

They are commonly referred to as remote loading strategies, facilitate the active entrapment of drugs within preformed liposomes by leveraging transmembrane chemical gradients. The pH gradient method capitalizes on a controlled difference in pH between the internal and external aqueous phases of liposomes to drive drug loading. Initially, blank liposomes are prepared via the thin-film dispersion method, after which their internal pH is adjusted to create a differential that promotes the diffusion of weakly acidic or basic drugs into the vesicle interior, where they become ionized and effectively trapped. This method allows for high drug-loading efficiency but is limited by its dependence on drug structure (Abraham et al. 2004).

1.3.4 Remote loading approach

Commonly used with the **ammonium sulfate gradient** method, which operates on the principles of chemical equilibrium. The blank liposomes are first hydrated with an ammonium sulfate solution, followed by dialysis against phosphate-buffered saline to establish a transmembrane ammonium sulfate gradient, wherein the internal concentration remains high while the external concentration is significantly lower. Upon incubation with a weakly basic

drug solution, ammonium ions facilitate drug encapsulation, leading to stable drug retention and prolonged storage stability (Bolotin et al. 1994). Similarly, the **calcium acetate gradient method** exploits a transmembrane concentration gradient to enhance drug loading efficiency. Blank liposomes encapsulating a calcium acetate solution are prepared using the thin-film hydration technique, after which external calcium acetate is removed through dialysis, creating a differential where the internal concentration remains elevated. This triggers the passive diffusion of acetic acid across the liposomal membrane, generating a pH gradient that facilitates the encapsulation of weakly acidic drugs. Once inside, these drugs interact with intravesicular calcium ions to form an insoluble calcium-drug complex, which prevents diffusion back across the membrane, thereby ensuring high entrapment efficiency and reducing premature drug leakage (Hwang et al. 1999).

These methodologies have significantly improved the efficacy of liposomal formulations, allowing for optimized drug delivery. Meanwhile for the better efficacy, therapeutic effects and consistency in the liposomal based preparation, the advanced technology such as **Microfluidics technology** has been making cornerstone of modern nanomedicine (Shah et al. 2019).

1.4 Microfluidics technology: State of art

Microfluidics, a field that manipulates small fluid volumes within microscale channels, is governed by fundamental principles of fluid dynamics, pressure-driven flow, and thermodynamic correlations, all of which contribute to the intricate behavior of liquid movement at these minuscule scales. The foundation of microfluidic flow lies in the low Reynolds number (Re) regime, which ensures a laminar flow profile, meaning that fluid particles move in parallel layers with minimal mixing, as opposed to turbulent flow found in larger-scale systems. The Reynolds number, a dimensionless quantity that characterizes the ratio of inertial forces to viscous forces within a flowing fluid, is defined as

$$Re = \frac{\rho u D_h}{\mu}$$

Where ρ represents fluid density, u is the characteristic velocity, D_h is the hydraulic diameter of the microchannel, and μ is the dynamic viscosity (Carugo et al. 2016). In microfluidics, due to the small characteristic dimensions and the dominance of viscous forces, Reynolds numbers typically remain below 100, reinforcing a strictly laminar flow regime, where molecular diffusion becomes the principal mechanism for mass transfer rather than chaotic convective mixing (Jahn et al. 2010). The absence of turbulence necessitates innovative mixing strategies, such as the integration of obstacles, staggered herringbone mixers (SHM),

and hydrodynamic focusing techniques, which induce localized chaotic advection, thereby enhancing diffusion-driven mixing (Kastner et al. 2014).

The Navier-Stokes equation, which governs the motion of fluids, simplifies significantly in microfluidic environments due to the negligible contribution of inertial forces, leaving a balance between pressure gradients and viscous forces (Cottet and Renaud 2021). The parabolic velocity profile, a hallmark of pressure-driven flows in microchannels, emerges due to the no-slip boundary condition at the channel walls, leading to a scenario where the fluid velocity is highest at the center and decreases to zero at the boundaries (Y. Li et al. 2005). Originally Poiseuille's law deals with the cylindrical pipe where the parabolic velocity profile follows a single equation across the cross-section. In rectangular microchannels which are widely used in microfluidics, the solution is derived from the Navier-Stokes equation in three dimensions, considering both width and depth (Bazaz et al. 2020). This equation emphasizes the dependence of flow velocity on channel geometry, reinforcing the idea that channel shape and dimensions play a critical role in fluid transport. The pressure-driven nature of microfluidic flow ensures that the velocity distribution follows a well-defined parabolic shape, where hydrodynamic resistance increases as the cross-sectional dimensions shrink, requiring higher pressures to maintain the same volumetric flow rate (Falk and Commenge 2010). The interplay between pressure differentials, viscosity, and channel dimensions dictates the overall fluid behavior, making precise control over flow conditions essential for applications such as liposome synthesis, drug delivery, and biochemical assays (Carvalho et al. 2022). Beyond the Reynolds number, additional dimensionless numbers help quantify various transport phenomena in microfluidic flows. The Péclet number (Pe), given by

$$Pe = \frac{uL}{D}$$

where D is the diffusion coefficient, u is mean velocity, L is the characteristic length characterizes the relative importance of advective transport to molecular diffusion. A high Péclet number ($Pe > 1000$) indicates that advection dominates, leading to poor mixing, whereas a low Péclet number ($Pe < 1$) signifies that diffusion is the primary transport mechanism, allowing for efficient mixing over short distances (Jeong et al. 2010). Similarly, the Knudsen number (Kn), which compares the mean free path of fluid molecules to the characteristic channel dimensions, plays a role in assessing the transition from continuum to rarefied flow, particularly in gas microfluidics. In most liquid-based microfluidic systems, Knudsen numbers remain low, ensuring that the fluid follows continuum mechanics, where the Navier-Stokes equations remain valid (H. Li, Ewoldt, and Olsen 2005). However, in nanoscale fluidics, where channel dimensions approach molecular scales, slip-flow and non-continuum effects become significant, requiring modifications to classical flow models (Squires and

Quake 2005). The thermodynamic interplay with microfluidic hydrodynamics is another critical aspect influencing fluid behaviour, particularly in temperature-dependent transport phenomena. The first law of thermodynamics, which governs energy conservation, dictates that heat generation due to viscous dissipation and wall-fluid interactions can influence microfluidic flow stability (Pinho et al. 2014). In microscale flows, Joule heating effects (resulting from electric fields in electrokinetically driven systems) and thermal gradients can induce Marangoni flows, where surface tension variations drive secondary convective currents. This is particularly relevant in applications involving thermal control of chemical reactions, biological assays, and droplet-based microfluidics, where precise temperature regulation is required (Bayareh, Ashani, and Usefian 2020). Furthermore, the second law of thermodynamics, which governs entropy and irreversibility, plays a role in understanding diffusion-driven mixing, where molecular transport seeks to maximize entropy, leading to gradual homogenization of solutes over time (Naterer and Camberos 2008). The integration of thermodynamic and hydrodynamic principles is crucial in the design of microfluidic heat exchangers, phase-change-based actuators, and bioanalytical devices, where energy transport mechanisms such as conduction, convection, and phase transitions are exploited for enhanced functionality. Mass transfer in microfluidic systems for the drug delivery system is fundamentally different from that in conventional bulk mixing. In macroscale systems, turbulence plays a dominant role in enhancing mass transfer by promoting chaotic mixing and convective diffusion. However, due to the small scale of microfluidic channels (typically ranging from micrometers to millimeters in width), flow remains laminar, and diffusion becomes the primary mechanism of mass transport (Nguyen and Wu 2004). The efficiency of mass transfer in microfluidics is governed by Fick's laws of diffusion, which describe how molecules move from regions of higher concentration to lower concentration. In systems where hydrodynamic focusing is employed, the solvent (e.g, ethanol or isopropanol) diffuses into the surrounding aqueous phase while water diffuses in the opposite direction. This process creates a controlled solvent exchange environment where amphiphilic molecules, such as phospholipids, reach their solubility threshold and self-assemble into vesicular structures (Castillo-León and Svendsen 2014). The geometrical design of microfluidic chips significantly influences flow characteristics, with variations such as T-junctions, serpentine channels, and converging-diverging structures altering flow resistance and mixing efficiency (Ansari, Kim, and Kim 2018; Clark, Kaufman, and Fodor 2018; Milotin and Lelea 2016). For instance, split-and-recombine (SAR) mixers leverage periodic flow division to enhance diffusion, while staggered herringbone mixers (SHM) utilize chaotic advection by inducing secondary rotational flows (Capretto et al. 2011). The hydrodynamic boundary conditions, including the relative flow rates (flow rate ratio, FRR) and total flow rate (TFR), play a decisive role in controlling droplet formation in two-phase flows, as seen in emulsification and nanoparticle

synthesis. By tuning the viscosity ratio between immiscible phases, researchers can achieve precise control over droplet size, shape, and generation frequency, making microfluidics an indispensable tool for applications in pharmaceutical formulations, lab-on-a-chip diagnostics, and high-throughput screening (Azizipour et al. 2020; Niculescu, Mihaiescu, and Grumezescu 2022; Stroock et al. 2002).

The synergy between fluid mechanics, thermodynamics, and microfluidic engineering underscores the interdisciplinary nature of microfluidics, where precise manipulation of pressure-driven flow, diffusion mechanisms, and thermally influenced transport enables ground-breaking advancements in biotechnology, nanomedicine, and point-of-care diagnostics. By leveraging fundamental principles of hydrodynamics and thermodynamics, researchers continue to push the boundaries of microfluidic technology, paving the way for more efficient, scalable, and application-specific innovations in biochemical analysis, drug formulation, and precision medicine (Zimmerman 2006).

2. Aim of Work

The primary aim of this research is to develop an optimized liposomal formulation for quercetin, harnessing the advanced capabilities of microfluidics technology. This work is directed toward the preparation of an oral liposomal supplement that enhances the bioavailability and therapeutic efficacy of quercetin, a flavonoid known for its potent anticancer and antioxidant properties. By employing microfluidic synthesis, the study endeavors to achieve precise control over critical formulation parameters such as the flow rate ratio (FRR) and the total flow rate (TFR), which are instrumental in dictating vesicle size, polydispersity, and overall stability of the liposomes.

The investigation focuses on optimizing these microfluidic conditions to produce liposomes that are within the ideal particle size range (approximately 100-150 nm) and exhibit low polydispersity indices, ensuring uniformity and reproducibility in formulation. Utilizing food-grade, FDA-approved excipients, such as soy lecithin and beta-sitosterol, the formulation is designed for oral administration, thereby addressing common challenges associated with conventional drug delivery systems, including poor solubility, erratic absorption, and rapid degradation.

In collaboration with Galenica Senese, the project aims not only to refine the microfluidic parameters but also to validate the safety profile of the developed liposomes through comprehensive in vitro studies, including cell viability assays on healthy lung fibroblasts (MRC-5). Ultimately, the goal is to establish a robust, scalable platform for the production of quercetin liposomes that combine enhanced stability, controlled release properties, and minimal cytotoxicity-qualities that are pivotal for both clinical research and eventual commercial application.

3. Materials and Methods

3.1 Materials

Quercetin dihydrate (>95% purity, MW: 338.32 g/mol) was provided by Farmalabor SRL. Soy lecithin (MW: 756 g/mol) lipid was gifted by Lipoid GmbH and food grade beta-sitosterol (MW: 414.7 g/mol), chloroform and ethanol absolute were purchased from Sigma Aldrich. Dulbecco's Phosphate Buffered Saline (DPBS) was bought from Merck. Water was purified with Milli-Q® (Millipak® 0.22 µm) system. Fasted state simulated intestinal fluid (FaSSIF) pH 6.5 without Enzyme Dissolution Media was purchased from Reagecon. Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were purchased from Thermo Fischer labs.

3.2 Intrinsic solubility analysis of Quercetin

Solubility of Quercetin was estimated in ethanol and water, procedure was in accordance with Higuchi and Connors, an excess amount of drug was added in 1 mL of the solvents (ethanol and water), the solution was vortexed, sonicated at 37 °C for 20 mins and was kept for 24 hours in the thermal shaker at 37 °C at 100 rpm to attain the equilibrium between the solvents. The measurements were recorded in triplicates. The samples were centrifuged at 37°C to maintain the equilibrium at the speed of 5000 g for 30 mins. To estimate the aqueous solubility of quercetin, the calibration curve of the quercetin in ethanol to water (1:1) was prepared and using the molar absorptivity from the equation obtained, the soluble concentration of quercetin was estimated. Meanwhile supernatant was collected of the quercetin/ethanol and both of the drug/solvent solubilities were estimated using UV/Vis spectroscopy at 374 nm.

3.3 Microfluidics Synthesis

To produce both the empty and quercetin loaded liposomes was applied a microfluidics setup composed by commercially available components: Microfluidics apparatus (Sunshine, Unchained labs, Milan, Italy) constitute of pressurized fluid bottles, 2 quad pumps (Organic and aqueous), automated sample valve and collector. The instrument has a microfluidics chip holder named as sunny rail where the microfluidic chip (Sunnies) is combined with the loop system (10 mL loop) present in the automated sample valve as shown in the Figure 2. In the experiment, glass microfluidics chip with T and X junctions with 190 µm etch depth was used. The chip is a laminar flow reactor exhibiting the pockets of chaotic advection when used under optimal conditions. The microfluidic device was controlled by the Sunny Suite Software which allowed to create protocols and automated experiments.

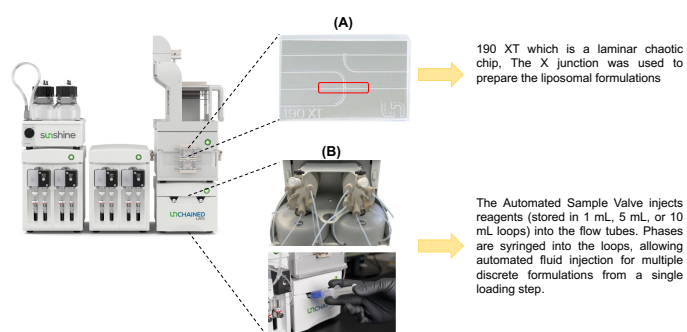


Figure 2 - Microfluidics setup. (A) Microfluidics chip with 190 μm etch depth and (B) Automated sample valve and loop system for the delivery of organic and aqueous phase.

Blank and quercetin-loaded liposomes were prepared using a lipid mixture of soy lecithin and β -sitosterol at molar ratios corresponding to 2.5 and 10 mM. Lipid concentrations were chosen to systematically explore how varying lipid availability impacts the self-assembly of liposomes in a microfluidic system. 2.5 mM represents a moderate concentration often used in microfluidic liposome research (0.1-3 mM), facilitating well-controlled mixing and size distribution (Chang and Yeh 2012). 10 mM, on the other hand, approaches the higher end of lipid loading, bridging the gap toward commercially relevant liposomal (5-25 mM) formulations (Allen and Cullis 2013). Initially, the lipid mixture was dissolved in ethanol to maintain the desired molar ratio, and the solution was heated to 60°C to ensure that the soy lecithin was above its phase transition temperature, thereby achieving a homogeneous, fluid state. For quercetin-loaded formulations, quercetin was incorporated into the ethanol solution at a concentration of 1 mg/mL, and the excipients quantities were adjusted according to the flow rate ratios (FRRs) required for subsequent microfluidic processing. Liposome formation was conducted using 190 XT chip provided by the Sunshine, Unchained Labs. FRR conditions of 3 and 5, with total flow rates (TFRs) varied between 6 and 12 mL/min, while a 10 mM DPBS aqueous phase was consistently used.

In the current experiments, the effective lipid concentration in the final liposomes is dictated primarily by the FRR rather than the TFR. For example, using an initial 10 mM organic solution, an FRR of 3 (where the aqueous phase is three times the volume of the lipid phase) dilutes the organic phase fourfold, resulting in an effective concentration of 2.5 mM. Therefore, to obtain the 10 mM as the excipient concentration in the final formulation, the excipients dissolved in ethanol solution should be 4 times more as an initial solution when preparing for FRR 3 and similarly, using a FRR of 5, the dilution factor would be 6, requiring a 60 mM initial solution to reach 10 mM in the final formulation.

Table 2 - Required initial and final lipid molarities (mM) for maintaining a 6:4 molar ratio of soy lecithin to β -sitosterol at target final total lipid concentrations of 2.5 and 10 mM using an FRR 3 and 5.

Soy Lecithin: β - sitosterol		
Flow rate ratio (FRR)	Initial Excipient Molarity (mM)	Final Excipient Molarity (mM)
3 (<i>dilution factor 4</i>)	10 (6:4)	2.5 (1.5:1)
	40 (24:16)	10 (6:4)
5 (<i>dilution factor 6</i>)	15 (9:6)	2.5 (1.5:1)
	60 (36:24)	10 (6:4)

The collected samples were dialyzed overnight against DPBS (10 mM) using the dialysis tubing cellulose membrane (14 kDa MWCO; Merck) to eliminate unencapsulated molecules and residual ethanol, thereby preventing lipid interdigitation within the bilayer and preserving the structural integrity of the liposomes.

3.4 Dynamic Light Scattering (DLS)

The particle size, polydispersity index (PDI), and zeta potential were examined by Zetasizer Nano particle analyser (Malvern Panalytical, Malvern, UK). The size was measured at 25°C, equilibrating time of 60 seconds. The samples were diluted from 1:10 to 1:100 to optimize the desired dilution for the estimation. DLS was conducted on all samples prepared under the varied conditions. Only those samples meeting the optimal criteria were selected for further analysis, while the remainder were excluded. Quercetin liposomes deterioration over storage was evaluated by DLS analysis over time up to one month. Samples were dispersed in DPBS and maintained at 4 °C.

3.5 Transmission Electron Microscopy (TEM)

A 25 μ L drop of the solution was placed onto a 400-mesh holey film grid. Following a 2-minute staining with 1% uranyl acetate, the samples were examined using a FEI Tecnai G2 transmission electron microscope (Hillsboro, Oregon, US) operating at 100 kV. Images were captured with a Veleta digital camera (Olympus Soft Imaging System) and processed using ImageJ software.

3.6 Phospholipid Quantification using Stewart assay

Lipid quantification was performed using the Stewart assay. The ferro thiocyanate reagent was prepared following the protocol described in (Prima, Librizzi, and Carrotta 2020): 27.03 g of ferric chloride hexahydrate (Sigma Aldrich) and 30.4 g of ammonium thiocyanate (Sigma Aldrich) were dissolved in double distilled water and brought to a final volume of 1 L; the resulting solution remains stable at room temperature for several months. In glass tubes, 1.5 mL of chloroform and 1.5 mL of the prepared Stewart reagent were added, and each condition was prepared in triplicate. For the calibration curve, 20 μ L of a blank lipid standard ranging

from 2 to 10 mM were added in the glass tubes and were prepared in triplicates. The assay, which relies on the formation of a colored complex between phospholipids and ammonium ferrothiocyanate in an organic solvent, was measured at 485 nm. After vortexing, the samples were centrifuged at 5000 rpm for 15 minutes at 20°C, and the supernatant was collected for phospholipid calibration curve using UV spectroscopy. To quantify the lipid concentration, 20 uL of the blank liposome was similarly added in another glass tube containing the chloroform and stewart reagent which was further vortexed and centrifuged using the similar conditions and supernatant was estimated.

3.7 Encapsulation efficiency (EE %) and Drug loading (DL %)

The quercetin liposomal samples (500 µL) were added in absolute ethanol (500 µL), vortexed and were bath sonicated at 50°C for 20 mins. The samples were collected and centrifuged at 17,000 g, 4°C for two hours with Micro Star 17R (Avantor, Radnor, Pennsylvania, USA). The supernatant was collected and diluted 1:10 with ethanol and concentration of quercetin was estimated at 374 nm using Cary 100 UV-Vis Spectrophotometer, Agilent.

3.8 In Vitro drug release

In vitro release tests were performed using 2.5 mM liposomal solution in pre-prepared FASSIF simulated intestinal fluid at pH 6.8 in case of gastric media the FASSIF fluid was pH adjusted to 1.6 by adding HCl. In each of the media 10% ethanol absolute added to maintain to quercetin solubility. The quercetin liposomes were added in the dialysis bag (MWCO, 5000 Da) and was immersed in a beaker containing 50 mL of the release media. The conditions were maintained at 37 °C and stirred (100 rpm). After predefined periods of 2, 4, 6, 24, 48, 96 hours samples were taken, measured using Cary 100 UV-Vis Spectrophotometer at 374 nm and replaced back in the same media to cancel out the sink condition calculations. The experiments were performed in triplicate.

3.9 Cytotoxicity analysis of the components and liposomes

The cytotoxicity on human lung fibroblasts (MRC-5), used as a model for normal cells, was assessed by evaluating soy lecithin (starting concentration of 200 μ M), beta-sitosterol (starting concentration of 1 mM), blank liposomes (starting concentration of 200 μ M), and free quercetin, with cisplatin serving as a positive control. MRC-5 cells were seeded in 96-well plates at a density of 8,000 cells per well. On the following day, the cells were treated with serial 1:2 dilutions of each compound in culture media. After the specified incubation period, cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571) on a Synergy H1 plate reader, and IC₅₀ values were calculated using GraphPad Prism software.

4. Results and Discussion

4.1 Solubility of Quercetin

Quercetin is predominantly hydrophobic due to its extensive π -conjugated ring system and the propensity for intramolecular hydrogen bonding among its multiple hydroxyl groups, which diminishes the molecule's capacity to form hydrogen bonds with water. In ethanol, the presence of both polar and nonpolar regions in the solvent facilitates stronger intermolecular interactions with quercetin's aromatic backbone and hydroxyl groups, thereby enhancing its solubility (Hollman et al. 1997).

Table 3 - Solubility of Quercetin. The quercetin hydrophobic nature can be expressed in the aqueous solubility value performed at pH 7.4 meanwhile it's high solubility in ethanol is explainable through the value represented, recorded using UV/Vis spectroscopy. The values are expressed in Mean $\pm$ SD (g/L).

Solubility of Quercetin $\pm$ SD (g/L)	
Ethanol	Water
39 $\pm$ 1.8	0.2 $\pm$ 0.002

4.2 Microfluidic optimization of Quercetin Liposomes

The production of blank and quercetin loaded liposomes was studied considering the influence over samples with different flow rate ratio (FRR 3 and 5), total flow rate ratios (6, 8, 10, 12 mL/min), and varied excipients concentration (2.5 and 10 mM). When preparing liposomes via microfluidic mixing, several interdependent parameters—flow rate ratio (FRR), total flow rate (TFR), total lipid concentration, and excipient composition—govern the nucleation and growth of lipid vesicles, ultimately affecting liposome size, polydispersity index (PDI), and stability.

At a 2.5 mM total excipient concentration, the key driver behind liposome size and uniformity is the rate at which the organic (lipid-containing) phase is diluted by the aqueous phase, which in microfluidics is controlled by the flow rate ratio (FRR). In laminar (low Reynolds number) microfluidic flows, the streams do not turbulently mix. Instead, mixing occurs largely by diffusion at the boundary between the organic and aqueous phases (Pittiu et al. 2024). Thus, in an X-junction chip (190 XT) which was used in the experiment, two aqueous inlets converge at one central organic inlet creating an interface where mixing occurs primarily by molecular diffusion rather than turbulent eddies (Yu, Lee, and Lee 2009). In the Figure 3, when the FRR at 3:1 (aqueous-to-organic), the lipid phase is diluted more quickly and uniformly upon contacting the aqueous phase (DPBS). Thermodynamically, this rapid dilution means lipids reach a supersaturation point faster and nucleate into bilayers almost immediately. Because nucleation happens in a relatively confined, homogeneous environment, the resulting vesicles tend to be smaller as represented in Figure 3 (A) and (B) the sizes range from 50-70 nm with a narrow PDI of 0.1-0.2. The results obtained maintained the similar size range and polydispersity under varied TFR, because once the flow rates are sufficiently high to maintain stable laminar conditions (but not so high as to induce channel instabilities), increasing the

TFR primarily speeds the fluid velocities through the chip channels without drastically changing the diffusion pathways or the time the two phases must interpenetrate. Consequently, if the FRR stays constant, the rapid dilution kinetics of the lipid-containing phase are effectively the same whether the total flow is 6 mL/min or 12 mL/min. These results are also in accordance with Roces, Carla B., et al. in comparable liposome sizes and similarly narrow PDIs across that TFR ranges (Roces et al. 2020).

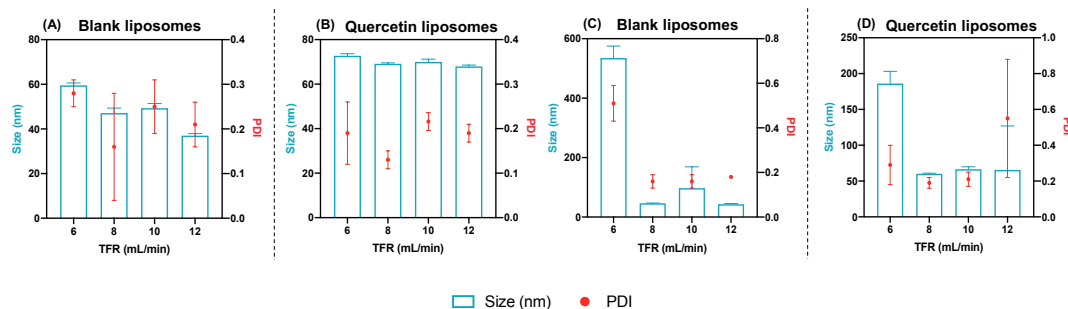


Figure 3 - Particle Size and PDI Analysis. Samples prepared with a 2.5 mM organic phase at an FRR 3 include: (A) Blank Liposomes and (B) Quercetin-loaded Liposomes, both produced at TFRs ranging from 6 to 12 mL/min. Similarly, samples synthesized at an FRR 5 comprise: (C) Blank Liposomes and (D) Quercetin-loaded Liposomes, each generated at TFRs of 6-12 mL/min.

In the theoretical perspective, increasing the FRR tends to yield smaller liposomes. The reasoning is straightforward: a higher aqueous fraction generally dilutes the lipid solution more rapidly, promoting earlier onset of supersaturation and thus nucleation of a larger number of smaller vesicles. In the practical scenarios the trend is influenced by multiple factors such as total lipid concentration, chip geometry, channel dimensions, flow rates, and fluid properties (viscosity and interfacial tension) (Carugo et al. 2016). When the FRR 5 was used (Figure 3 B) at TFR 6 mL/min showed the particle size of 186.1 ± 17 nm with PDI of 0.3 ± 0.1 could be due to the stretching or deformation of the mixing interface creating local pockets of differing solvent composition. The improper mixing can lead to precipitation of the lipids in the mixing channels producing larger or more heterogeneous liposomes. If the channel cannot provide adequate residence time at higher FRRs, nucleation occurs under suboptimal conditions. Meanwhile at TFR 8 and 10 mL/min, fluid viscosity become higher and there is more effective chaotic or diffusive mixing within the same channel geometry. This enhanced mixing environment accelerates and homogenizes lipid supersaturation and vesicle nucleation, resulting in smaller (60-66 nm) and more monodisperse (PDI 0.1-0.2) liposomes. On the other hand, TFR 12 mL/min the deviation obtained with the particle size was observed high. According to (Carugo et al. 2016) the TFRs at certain higher limit can induce high flow velocity, reduced residence time, and local shear stresses in the mixing channels. In the current experimental setup, a TFR of 12 mL/min appears to exceed the optimal range for effective mixing. Although increasing the flow rate can improve mixing up to a certain point, pushing the TFR too high inadvertently introduces unfavourable conditions that undermine vesicle size control.

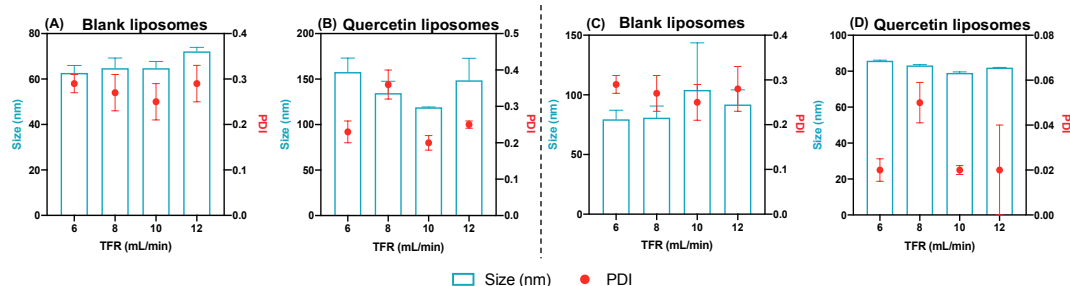


Figure 4 - Particle Size and PDI Analysis. Samples prepared with a 10 mM organic phase at an FRR 3 include: (A) Blank Liposomes and (B) Quercetin-loaded Liposomes, both produced at TFRs ranging from 6 to 12 mL/min. Similarly, samples synthesized at an FRR 5 comprise: (C) Blank Liposomes and (D) Quercetin-loaded Liposomes, each generated at TFRs of 6-12 mL/min.

When the total lipid concentration is increased to 10 mM, there is substantially more lipid available to form bilayers, which alters both the thermodynamics of vesicle nucleation and the fluid physics within the microchannels. In an X-junction chip with two aqueous inlets and one central organic inlet, mixing depends primarily on controlled, laminar diffusion between the streams. At FRR = 3, the organic phase constitutes a larger fraction of the total flow, so lipid precipitation into vesicles is comparatively slower and occurs under a lipid-rich environment. Consequently, liposome sizes as represented in the Figure 4 (B) at TFRs of 6, 8, and 10 mL/min (157.8 ± 15.32 nm, 134.6 ± 13.05 nm, and 119 ± 0.29 nm, respectively) are generally larger than what is typically observed at lower lipid concentrations (2.5 mM). As flow rates increase within this higher-concentration regime, diffusion-based mixing accelerates but can still leave zones of more concentrated lipid, thereby explaining why particle size eventually rose again to 148.6 nm at 12 mL/min: overshooting the optimal mixing velocity can reduce the residence time needed for uniform dilution. In contrast, at FRR = 5 in Figure 4 (D) the aqueous phase dilutes the organic phase more aggressively, maintaining a more uniform solvent environment that favors smaller, more homogeneous liposomes (approximately 80-85 nm) regardless of TFR between 6 and 12 mL/min. From a thermodynamic standpoint, the higher initial lipid concentration (10 mM) intensifies the supersaturation threshold once the organic stream meets the aqueous streams, but the relative dilution by FRR and shear flow of TFR still dictate whether lipids condense into fewer, larger vesicles or numerous, smaller ones. The laminar, diffusion-driven nature of the X-junction means that both FRR and TFR must be carefully balanced, even slight imbalances can create compositional “pockets” where nucleation and growth conditions differ.

In comparing these outcomes to those observed at 2.5 mM, the lower concentration naturally predisposes the system toward smaller, more uniform liposomes across a broader range of flow conditions. With fewer lipid molecules available, oversaturation events are more easily controlled, and the process is less sensitive to subtle variations in mixing. Thus, while 2.5 mM can yield stable and relatively small vesicles under a wide window of FRR and TFR values,

pushing the concentration to 10 mM demands tighter control of fluid flow parameters to achieve the desired liposome size and maintain low polydispersity.

From the extensive set of experiments conducted, two specific liposomal formulations emerged as particularly promising based on their therapeutic potential. One formulation, prepared at 2.5 mM lipid concentration with an FRR of 3 and a TFR of 8 mL/min, yielded smaller vesicles deemed ideal for the clinical research. In parallel, the 10 mM formulation under the same FRR and TFR conditions produced larger liposomes, which are more suitable for oral delivery as a liposomal supplement. These selected conditions were then re-created to verify reproducibility, and further characterizations were carried out to confirm their stability and efficacy.

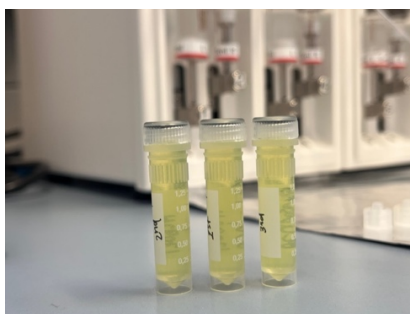


Figure 5 - Freshly prepared Quercetin liposomal samples using Sunshine microfluidics at FRR 3 and TFR 8 mL/min using 2.5 mM and 10 mM.

4.3 Quercetin Liposomes characterization

4.3.1 Dynamic Light Scattering (DLS)

When formulating quercetin-loaded liposomes via microfluidics at FRR 3 and TFR 8 mL/min, the difference in lipid concentration (2.5 mM vs. 10 mM) plays a pivotal role in dictating both particle size and polydispersity index (PDI). At a lipid concentration of 2.5 mM, fewer lipids are available to form each bilayer, resulting in smaller vesicles of 98.5 ± 11.4 nm and a mono dispersity at $PDI 0.27 \pm 0.02$. In contrast, increasing the lipid concentration to 10 mM provides a greater amount of lipid to drive vesicle formation, often leading to slightly larger liposomes (110 ± 4 nm) and a broader distribution ($PDI 0.33 \pm 0.007$) as presented in the Figure 6 (A). Moreover, the presence of β -sitosterol within the soy lecithin membrane can enhance membrane packing and possibly heighten the likelihood of transient coalescence events, which further contributes to the observed increase in size and polydispersity.

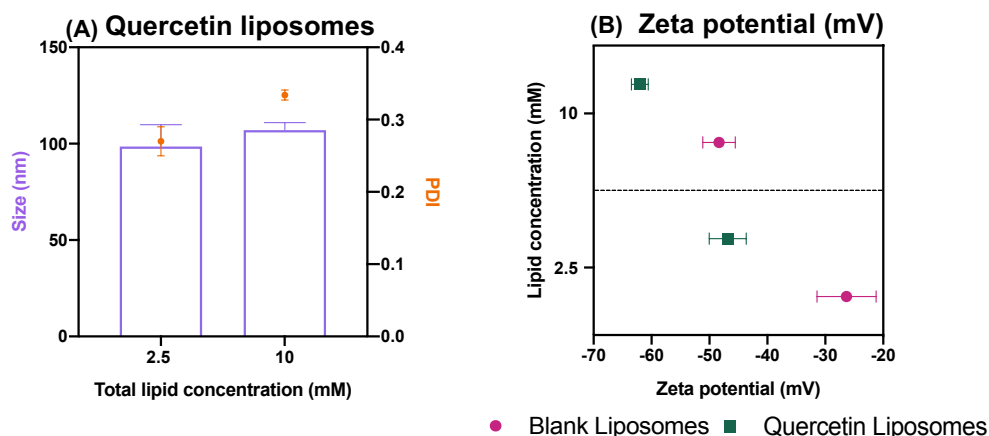


Figure 6 - Particle size, polydispersity index (PDI), and zeta potential of blank and quercetin-loaded liposomes formulated at 2.5 mM and 10 mM lipid concentrations. (A) Bars (left) represent the mean particle size $\pm$ SD, while the orange markers show the corresponding PDI. (B) The green and pink markers (right) indicate the zeta potentials of blank and quercetin-loaded liposomes, respectively. All measurements were performed in triplicate.

Zeta potential measurements (Figure 6B) reveal similar concentration-dependent behaviour. At higher lipid concentrations, there are more negatively charged phospholipid head groups per liposome, leading to a more negative baseline zeta potential. Hence, blank liposomes at 2.5 mM display a value of -26.33 mV, compared to -48.35 mV at 10 mM. The incorporation of quercetin, which contains phenolic groups that can deprotonate under certain conditions, augments the surface charge, driving the zeta potential to even more negative values (Amorati et al., 2017). Specifically, quercetin-loaded liposomes at 2.5 mM exhibit a zeta potential of -47 mV, while those at 10 mM reach -62.02 mV. This pronounced shift arises from quercetin's propensity to associate with the phospholipid head groups, exposing additional negative charges and reorganizing the membrane to accentuate its overall negative surface potential.

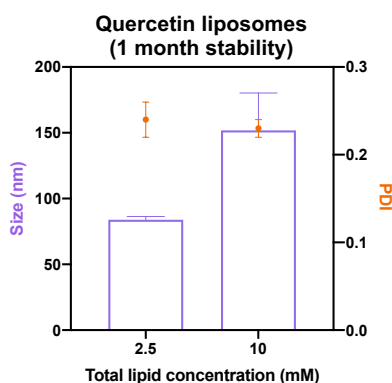


Figure 7 - Stability over 1 month of quercetin liposomes prepared using the varied lipid concentration of 2.5 mM and 10 mM.

When liposomal suspensions of varying lipid concentrations are stored at 4 °C, their stability can diverge due to differences in vesicle architecture and the overall lipid rearrangements. In the 2.5 mM formulation in the Figure 7, the liposomes are predominantly smaller and unilamellar, which tend to remain stable with minimal changes in size (83.93 ± 2.47 nm) because there is less total lipid mass that can reorganize over time. On the other hand, 10 mM

preparation contains larger vesicles, often multilamellar, meaning they have multiple concentric bilayers and a greater reservoir of lipid. During the one-month storage, these lipid bilayers can gradually undergo structural reorganization due to change in the phase transition temperatures, leading to a notable increase in average vesicle size from 108-152 nm. Even though the polydispersity index (PDI) remains relatively constant, indicating that the liposome population remains broadly similar in distribution. Individual vesicles may merge or expand enough to shift the overall hydrodynamic diameter upward. In essence, higher lipid concentration and multilamellarity make the liposome suspension more susceptible to slow fusion or layering phenomena at 4 °C, while the sparser, smaller vesicles at 2.5 mM are less prone to such transformations over the same period.

4.3.2 Transmission Electron Microscopy (TEM)

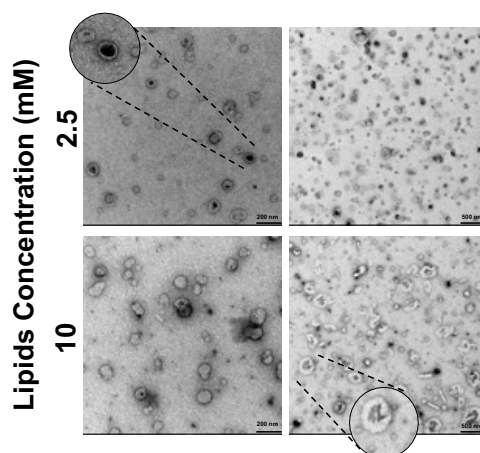


Figure 8 - TEM analysis of quercetin loaded formulations showing the spherical vesicles alongside highlighting the quercetin rich area in 2.5 mM and ring-like-structure in 10 mM.

TEM images of the liposomal formulations reveal distinct morphological differences between the samples prepared at 2.5 mM and 10 mM lipid concentrations. In the first set of images (2.5 mM), the liposomes appear predominantly as smaller, well-defined spherical vesicles with visible bilayer structures. Their relatively uniform shape and diameter, along with only slight size variations, agree with the dynamic light scattering (DLS) results indicating a narrower distribution. In the images there is a visibility of a black spot at 200 nm scale and 500 nm scales, there can be seen a bunch of particles. This is due to the electron- dense area where quercetin has aggregated or precipitated within the vesicle, resulting in a higher local density thus appears darker. In negatively stained samples, any regions of concentrated material—such as entrapped drug, or aggregated lipid can show up as darker “dots.” Thus, the black spot that can be seen in the Figure 8 surrounded by the bilayer is probably a quercetin-rich core rather than an imaging artifact (Román-Aguirre et al. 2020). Conversely, the 10 mM formulation exhibits larger vesicles with a broader range of particle sizes, which also corroborates the DLS findings of higher mean diameter and increased polydispersity. Many of

the liposomes in the 10 mM concentration (Melchior et al. 2023) display multilamellar characteristics. At the scale of 500 nm in the Figure 8, those “cushion-like” outer layers are likely multiple concentric lipid bilayers, often referred to as a multilamellar structure, surrounding the inner aqueous core or a quercetin-rich domain. At higher lipid concentrations (10 mM), (Román-Aguirre et al. 2020) reported that liposomes are more prone to forming these onion-like architectures, where successive layers of phospholipids encapsulate smaller interior volumes. The darker region in the center could represent aggregated quercetin or simply the internal aqueous compartment, but the overall appearance with multiple ring-like layers on the periphery is a characteristic of multilamellar vesicles observed under TEM.

4.3.3 Encapsulation efficiency (EE %), Phospholipid quantification and Drug loading (DL %)

Table 4 - Encapsulation efficiency (EE%) and Drug Loading (DL%) of quercetin loaded liposomes prepared using the lipid concentration of 2.5 mM and 10 mM.

Lipid Concentration (mM)			
2.5		10	
EE %	DL %	EE %	DL %
66 ± 1.0	29 ± 0.6	74 ± 1.1	15 ± 0.3

The data in the Table 4 clearly illustrate the contrasting effects of lipid concentration on EE % and DL% in liposomal formulations. The encapsulation efficiency is estimated using the formulation:

$$EE \% = \frac{\text{Amount of quercetin estimated}}{\text{Initial amount of quercetin added}} \times 100$$

Drug loading was determined based on the total lipid concentration. Prior to this calculation, the phospholipid content of the vesicles was quantified using the Stewart assay to accurately measure the lipids that formed the vesicular structures. A calibration curve was prepared, yielding a linear equation ($y = 45.259x$) with a regression coefficient of 0.9991, as illustrated in Figure 9. The slope ($\epsilon = 45.259$) was then used to calculate the phospholipid content in the liposomes. For a total lipid concentration of 2.5 mM, where the excipient was present at a 1.5:1 molar ratio (soy lecithin: β -sitosterol), the measured phospholipid concentration was 1.2

mM. In contrast, for a total lipid concentration of 10 mM with a 6:4 molar ratio, the observed concentration was 5.43 mM.

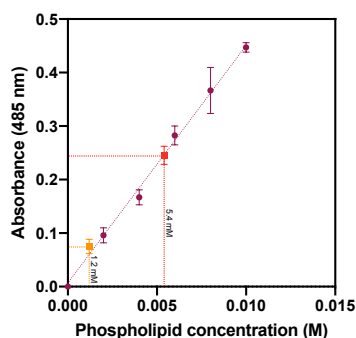


Figure 9 - Phospholipid Calibration Curve: The graph depicts the linear correlation between absorbance at 485 nm and lipid concentration (M) shown by dark red colour trendline. The linear equation obtained was $y = 45.259x + 0.0006429$ with $R^2=0.9991$. Red square points represent phospholipid quantification at a 10 mM total concentration (5.43 mM), whereas the orange square indicates the measurement obtained at a 2.5 mM total concentration (1.2 mM).

The drug loading was hence calculated using:

$$DL \% = \frac{\text{Molar concentration of quercetin}}{\text{Molar concentration of lipids}} \times 100$$

During the preparation quercetin was added in the same concentration of 1 mg/mL in the both the organic phases (2.5 and 10 mM), hence the effect in DL differed. Liposomes prepared at 10 mM exhibit a higher EE ($74 \pm 1.1\%$) compared to those at 2.5 mM ($66 \pm 1.0\%$), indicating that a greater fraction of the total quercetin is encapsulated when more lipid is available. This is attributable to the increased number of lipid molecules, which form larger vesicles that can sequester more of the drug overall. However, this enhanced entrapment comes at a cost: the absolute increase in encapsulated quercetin is offset by a disproportionately higher total lipid mass, resulting in a lower DL ($15 \pm 0.3\%$ at 10 mM versus $29 \pm 0.6\%$ at 2.5 mM). Essentially, while the higher lipid concentration facilitates more complete drug encapsulation, the quercetin becomes diluted across a larger amount of lipid, thereby reducing the relative proportion of drug per unit mass of lipid (Melchior et al. 2023). Moreover, the formation of larger and sometimes multilamellar vesicles at 10 mM further contributes to this effect, as additional lipid layers trap the drug but also add to the overall lipid content. In the current experiment, the EE % and DL% comparatively are higher than the reported in literature (Melchior et al. 2023) which can be achieved by the microfluidics technology.

4.3.4 In Vitro drug release

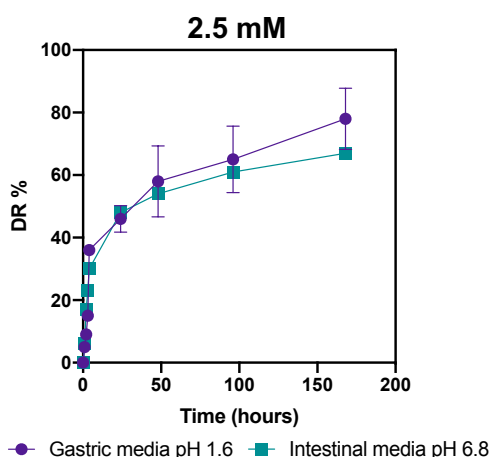


Figure 10 - In- vitro drug release of quercetin liposomes performed in gastric media of pH 1.6 as represented by the eggplant colour curve and intestinal media of pH 6.8 as represented by the teal-coloured curve maintaining the sustained release.

Quercetin liposomes formulated at a 10 mM lipid concentration reliably achieved particle sizes between 150 and 300 nm—an optimal range for oral delivery. This specific dimension is crucial, as it promotes efficient gastrointestinal absorption and interaction thus them liposomes (10 mM) were selected over quercetin liposomes prepared by 2.5 mM concentration. In the present study, the in vitro drug release profile of quercetin liposomes demonstrated a sustained release pattern that is particularly promising for oral administration. The formulation released quercetin gradually over an extended period, thereby maintaining its consistency, a factor that is crucial for enhancing bioavailability and therapeutic efficacy. As shown in Figure 10 an initial burst release of quercetin was observed within the first 6 hours, followed by a controlled and sustained release extending through 24 hours primarily due to the fraction of quercetin that is either adsorbed on or loosely associated with the surface of the liposomes. Quercetin liposomes showed this biphasic release behaviour is influenced by factors such as particle size, lipid composition, and encapsulation efficiency. Especially in the case of liposomes prepared using 10 mM lipid concentration that showed the ring like multilamellar structure is one of such contributing factors. In gastric media (pH 1.6) the release obtained was $78 \pm 10\%$ compared to the pH 6.8 intestinal media at $67 \pm 0.04\%$. This phenomenon can be primarily attributed to the pH-dependent solubility of quercetin. In acidic conditions, the solubility of quercetin is slightly increased, which, in conjunction with its altered partitioning behaviour, results in a higher concentration of the free drug available in the surrounding medium. Furthermore, the acidic environment appears to intensify quercetin's interaction with the liposomal membrane. At lower pH levels, the hydrogen bonding between quercetin and the phospholipid head groups is enhanced, leading to significant membrane perturbation. This interaction increases both the conductance and the electrical capacitance of the lipid bilayer, suggesting a destabilization that facilitates the diffusion of quercetin out of the liposomes

(Movileanu, Neagoe, and Flonta 2000). Particle size also plays an essential role in the release kinetics. Smaller vesicles, as observed in the 2.5 mM formulation, exhibit a higher surface area-to-volume ratio, which promotes a more efficient release of the encapsulated drug. Even larger or multilamellar vesicles, typically associated with more robust drug encapsulation, demonstrate increased permeability in the acidic environment due to the membrane disruptions.

4.4 Cytotoxicity analysis of the components and liposomes

The cytotoxicity analysis conducted on healthy MRC5 cell lines revealed that all components of the formulation which are quercetin, blank liposomes, lecithin, and β sitosterol exhibited IC₅₀ values greater than the starting concentration. This result in the Table 5 indicates that at the concentrations tested, each component and the overall liposomal formulation maintain a high degree of biocompatibility with minimal cytotoxic effects.

Table 5 - Cytotoxicity analysis expressed in IC₅₀ (μ M) included quercetin, soy lecithin, β -sitosterol, and blank liposomes on healthy lung-derived MRC-5 cells.

Components	IC ₅₀ (μ M)
Quercetin	> 100
Soy Lecithin	> 1000
β sitosterol	> 200
Blank Liposomes	> 200
Cisplatin	1.75 $\pm$ 0.21

There are several plausible reasons for the observed low toxicity of the components and liposomes on MRC5 cells. First, the formulation utilizes biocompatible materials: lecithin is a naturally occurring phospholipid integral to cell membranes, and beta-sitosterol is a plant-derived sterol known for its low toxicity profile. These components are inherently well-tolerated by healthy cells. Soy lecithin is generally generally recognized as safe (GRAS) which is primarily extracted from soybean seeds, has a rich blend of phospholipids, most notably phosphatidylcholine along with triglycerides, fatty acids, sterols, and glycolipids. As a dietary supplement, soy lecithin provides essential fatty acids and choline, a nutrient vital for neurotransmitter synthesis and overall cellular health. These components are instrumental in maintaining healthy lipid metabolism and supporting cognitive functions, while also contributing to a reduction in cholesterol levels. As they are derived from the natural food source it is inherently biocompatible, largely because its phospholipid components closely resemble those found in human cell membranes. This similarity facilitates its safe integration into the body. As a usage in supplement formulations, the range of 1-3 grams per day are

common and considered safe. Upon ingestion, the amphiphilic nature of soy lecithin aids its efficient adsorption by the gastrointestinal tract; it becomes incorporated into the lipid bilayer of cells or is metabolized into its constituent parts, such as fatty acids and choline. This gentle processing minimizes any potential cellular disruption, ensuring that even at relatively high concentrations, its cytotoxicity remains negligible (Alhajj et al. 2020). On the other hand, β -sitosterol is a phytosterol which is a plant-derived sterol structurally similar to cholesterol but distinguished by an additional ethyl group at the C24 position. It is predominantly found in a wide range of plant-based foods such as vegetable oils, nuts, seeds, cereals, and legumes. Thus, β -sitosterol is generally regarded as having low toxicity to healthy cells. This low toxicity is partly due to its limited absorption in the gastrointestinal tract; typically, only about 5-10% of dietary β -sitosterol is absorbed, with the majority being excreted. This inefficient absorption minimizes systemic exposure, which in turn reduces the risk of toxic effects (Ju et al. 2004). Moreover, encapsulating quercetin within liposomes can further reduce its potential toxicity. The liposomal carrier acts as a protective barrier, modulating the release of quercetin and preventing rapid, high local concentrations that might otherwise lead to cellular stress or damage. This controlled release helps maintain cellular homeostasis and minimizes exposure to potentially harmful peak concentrations.

5. Conclusion

This study successfully demonstrates the transformative potential of microfluidics technology in engineering quercetin-loaded liposomes for oral drug delivery. By carefully optimising the flow rate ratio and total flow rate, the research achieved precise control over liposome characteristics including particle size, polydispersity, and stability which are essential for ensuring therapeutic efficacy and bioavailability.

A key finding of this work is that liposomes prepared with a 10 mM lipid concentration consistently attained particle sizes in the 150-300 nm range, which is ideal for oral delivery. This size range is critical because it facilitates optimal absorption and interaction with the gastrointestinal tract, thereby enhancing the bioavailability of the encapsulated quercetin. Consequently, the *in vitro* release studies were conducted using the 10 mM quercetin liposomes to evaluate the drug release profile in both gastric and intestinal media. The sustained release observed under these conditions underscores the potential of the formulation as an effective oral supplement.

The incorporation of food-grade, FDA-approved excipients such as soy lecithin and beta-sitosterol further bolsters the biocompatibility and safety profile of the developed liposomal system. Cytotoxicity assays confirmed minimal toxicity on healthy cell lines, while the controlled release studies validated the ability of the system to maintain consistent drug delivery over extended periods.

Looking ahead, future work is expected to focus on scaling up the production process and conducting comprehensive *in vivo* studies to further validate the therapeutic potential and safety of the optimized liposomal formulations. Additionally, exploring the encapsulation of other bioactive compounds using similar microfluidic strategies could broaden the applicability of this technology, ultimately paving the way for more efficient, targeted, and patient-friendly therapeutic strategies in nanomedicine.

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Disclaimer

This manuscript has undergone grammar and syntax correction using ChatGPT, an AI language model developed by OpenAI. While every effort has been made to improve the clarity and accuracy of the language in this document, the authors acknowledge that the final content and scientific interpretations remain the sole responsibility of the authors and their collaborators. ChatGPT has been used exclusively to enhance the manuscript's readability and expression, and not to generate any type of data.

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Chapter 3

Microfluidic-Based Development of VS10-Loaded Liposomes for Targeted Pin1 Inhibition

1. Introduction

1.1 Ovarian Cancer

Ovarian cancer ranks as the fourth leading cause of cancer-related mortality in developed nations (Jayson et al. 2014; Reid, Permuth, and Sellers 2017). According to the Global Cancer Observatory over 300,000 new ovarian cancer cases and more than 190,000 deaths were recorded worldwide in 2020 (Sung et al. 2021). The asymptomatic nature of early-stage ovarian cancer often results in delayed diagnoses, contributing to its high mortality rate—over 75% of cases are identified only at advanced stages when the cancer has already spread beyond the ovaries and peritoneal cavity (Charkhchi et al. 2020; Lheureux, Braunstein, and Oza 2019). While early-stage ovarian cancer typically presents no symptoms and advanced-stage symptoms remain nonspecific (Doubeni, Doubeni, and Myers 2016), risk factors for the disease include both genetic and nongenetic components. Notably, mutations in the BRCA1 and BRCA2 tumor suppressor genes represent the predominant genetic risk factor, accounting for approximately 10% of ovarian cancer cases (Doubeni et al. 2016). Mutations in key tumor suppressor genes play a critical role in the hereditary development of both breast and ovarian cancers (Pruthi, Gostout, and Lindor 2010), and several nongenetic factors—including aging, obesity or weight gain, and the use of postmenopausal hormone therapy—further elevate the risk (CH 2000; Hunn and Rodriguez 2012).

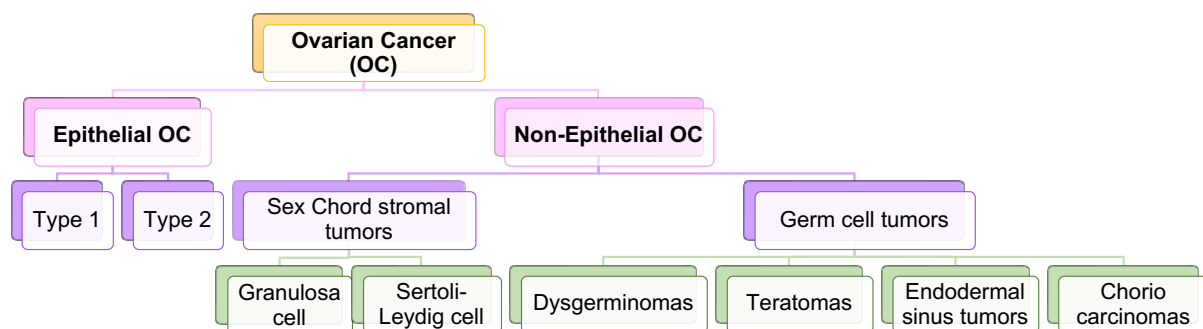


Figure 1 - Types of Ovarian cancer (OC), subdivided into epithelial and non-epithelial OC based on the cell of origin which are further subdivided into histopathological evaluation and molecular genetic analysis (Boussios et al. 2016).

Ovarian cancer is broadly classified into two major groups: non-epithelial and epithelial in Figure 1. Non-epithelial ovarian cancers, although less common (represents 10% ovarian cancer), comprise a diverse group of malignancies that arise from ovarian cells other than the surface epithelium; this group includes sex cord-stromal tumors (such as granulosa cell tumors and Sertoli-Leydig cell tumors, which originate from the ovary's supportive tissues and often produce hormones leading to distinctive endocrine symptoms), germ cell tumors (which develop from primordial germ cells and predominantly affect younger women—with examples including dysgerminomas, teratomas, endodermal sinus tumors, and choriocarcinomas that

are typically very responsive to chemotherapy when diagnosed early), and other tumors that are not otherwise specified (Boussios et al. 2016). In contrast, epithelial ovarian cancer (EOC) is further subdivided based on histological characteristics into types such as transitional cell, mucinous, clear cell, and serous tumors (Kurman 2013; Zhang et al. 2016; van Zyl, Tang, and Bowden 2018). In 2016, Kurman and Shih proposed a dualistic model in Figure 2 that refined the classification of EOC by integrating traditional histopathologic criteria with modern molecular genetic insights, thereby enhancing both diagnostic accuracy and prognostic predictions. According to this model, Type 1 EOC tumors are typically low-grade, harboring mutations in genes such as K-RAS, BRAF, and PTEN; they tend to exhibit genetic stability, progress slowly, and generally lead to favorable outcomes following surgical removal (Kurman 2013; Zhang et al. 2016; van Zyl et al. 2018). Conversely, Type 2 EOC tumors are high-grade, invasive, and aggressive, frequently associated with mutations in the TP53 gene and, in about 20–40% of high-grade serous ovarian cancers, somatic alterations in the BRCA1/2 genes—genetic aberrations that contribute to their rapid progression and poorer prognoses. Together, these insights underscore the importance of precise pathological evaluation and personalized treatment strategies to optimize outcomes in ovarian cancer management (Petersen et al. 2020; Petrucelli, Daly, and Pal 2022).

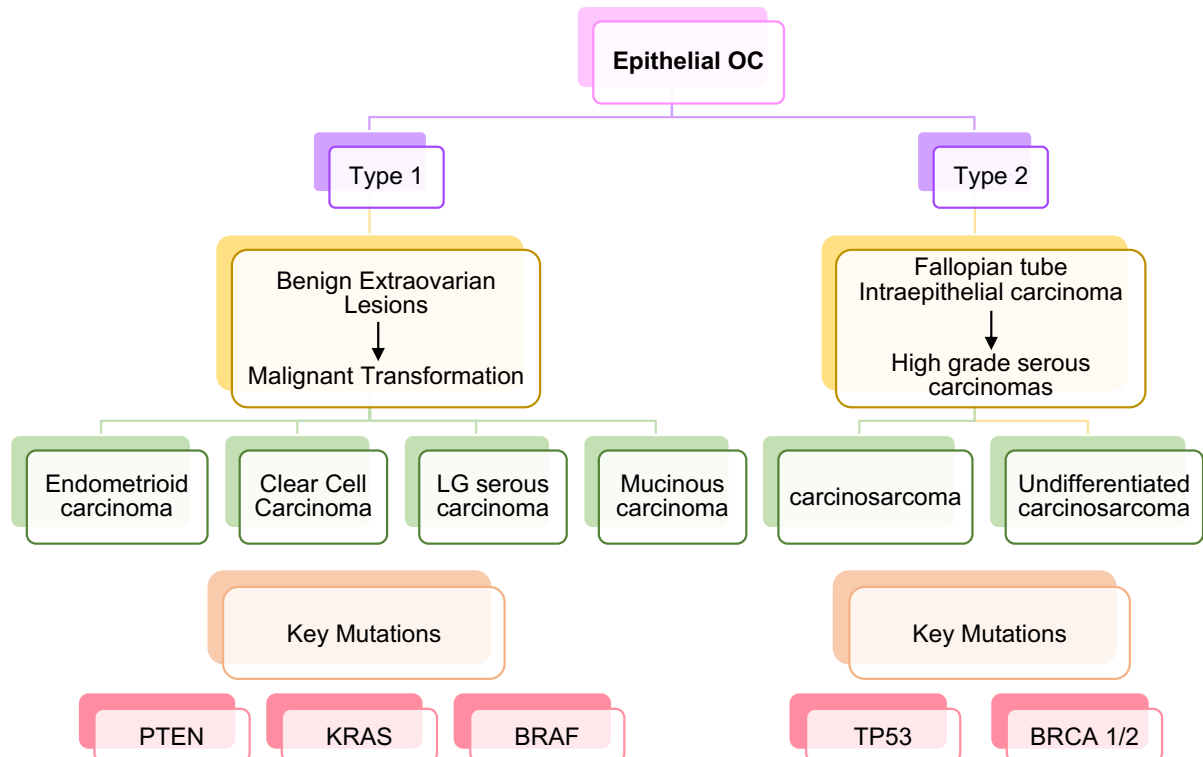


Figure 2 - The dualistic mechanism of epithelial ovarian cancer. Type I carcinomas comprise endometrioid, clear cell, LG serous, and mucinous carcinomas. Type II carcinomas are largely composed of HG serous carcinoma, carcinosarcoma indicating the genetic mutations (Kurman 2013).

High-grade serous ovarian cancer (HGSOC), classified as a Type II epithelial ovarian cancer (EOC), is the most aggressive subtype, accounting for 67% of all ovarian cancer cases and 70–80% of ovarian cancer–related deaths (Kurman 2013; Lisio et al. 2019; Zhang et al. 2016). According to the 2014 WHO classification, Type I EOCs—considered low-grade—include endometrioid, clear cell, seromucinous, low-grade serous, mucinous, and Brenner carcinomas, collectively responsible for only 10% of deaths. By contrast, Type II EOCs (e.g., carcinosarcomas, high-grade serous, and undifferentiated carcinomas) are designated high-grade and are characterized by pronounced aggressiveness and rapid development. Although the standard treatment approach for these patients involves cytoreductive surgery followed by chemotherapy, overall survival remains limited, and most eventually succumb to the disease (Chen et al. 2020; Kurman 2013; Scott et al. 2013). Furthermore, Type I and Type II tumors are believed to originate outside the ovary. Clear cell and mucinous EOCs are hypothesized to derive from endometrial stem cells (potentially linked to endometriosis), whereas high-grade serous carcinomas are postulated to arise from the epithelium of the fallopian tube (Chen et al. 2020; Scott et al. 2013).

1.2 Therapies in High Grade Serous Ovarian Cancer

High-grade serous ovarian cancer treatment has evolved from solely relying on surgery and platinum-based chemotherapy (Du Bois et al. 2009; Vergote et al. 2010) to a more personalized, targeted approach that integrates frontline maintenance options like PARP inhibitors (olaparib, niraparib, rucaparib) (Ashworth 2008) and angiogenesis inhibitors (bevacizumab, pazopanib) with emerging immunotherapies (Du Bois et al. 2014; Tewari et al. 2019), tyrosine kinase inhibitors (Tuefferd et al. 2007), and other novel agents in Table 1. In brief, while traditional surgery and chemo remain the backbone of initial treatment, maintenance strategies with targeted therapies have shown improved progression-free survival, and combinations—such as PARP inhibitors with VEGF blockade or immune checkpoint inhibitors—are being explored to overcome resistance and improve outcomes (Ray-Coquard et al. 2019). Although single-agent immunotherapy has shown modest results, multi-agent combinations and therapies targeting p53 reactivation, ribosome biogenesis, miRNA regulation, enzymatic activity, gene therapy, vaccines, and monoclonal antibodies are under investigation in both clinical and preclinical settings, each associated with distinct adverse effects ranging from myelosuppression, hypertension, and gastrointestinal toxicity to immune-related events (Dinkins et al. 2024).

Table 1 - Comprehensive overview of the targeted therapies being explored for high-grade serous ovarian cancer, categorizing agents based on their mechanism of action and clinical application. It covers frontline and secondary treatments, their typical use setting (clinical vs. preclinical), regulatory status, and key adverse effects or notable clinical considerations.

Targets	Targeting Agents	Treatment setting	Clinical/Preclinical Status	Common Side Effects	References
PARP Inhibitors	Olaparib, Niraparib, Rucaparib, Veliparib, Talazoparib	Frontline maintenance; recurrent disease in BRCA/ (Homologous recombination-deficient) HRD patients	Multiple phase III approvals (olaparib, niraparib); others in trials	Myelosuppression, nausea, fatigue, rare pneumonitis, secondary malignancies	(Lee and Matulonis 2020)
Angiogenesis Inhibitors (VEGF overexpressed)	Bevacizumab, Pazopanib, Apatinib	Combined with chemotherapy in frontline/recurrent disease	Approved in frontline and recurrent disease; various TKIs in trials (Trebananib)	Hypertension, proteinuria, GI perforation, thromboembolism	(Skorda et al. 2022)
Immunotherapy (PD-L1, CTLA-4 & MAbs)	Pembrolizumab, Nivolumab, Avelumab, Ipilimumab, Durvalumab, Tremelimumab	Recurrent disease; combination strategies	Limited single-agent efficacy; ongoing trials with combinations	Immune-related adverse events (colitis, dermatitis, endocrinopathies)	(Bachmann 2023; Gadducci and Guerrieri 2017)
Tyrosine Kinase Inhibitors	EGFR inhibitors (e.g., trastuzumab for HER2), FGFR inhibitors, PI3K-AKT inhibitors, MAPK-ERK inhibitors, AXL inhibitors	Targeting growth and survival pathways in resistant/recurrent disease	Mixed results in clinical trials; several agents in early/phase II trials	Rash, diarrhea, fatigue, variable toxicity profiles	(MRC Protein Phosphorylation and Ubiquitylation Unit n.d.; Skorda et al. 2022; Tuefferd et al. 2007)
P53 Reactivators	APR-26, ReACp53 (peptide blocking amyloid), carboplatin/doxorubicin	Restoring wild-type p53 function in mutated tumors	Early-phase trials and preclinical studies (combination therapies)	Generally well tolerated; efficacy and long-term safety still under evaluation	(Mohell et al. 2015; Neal et al. 2021; Ovejero-Sánchez, González-Sarmiento, and Herrero 2023)
miRNA-Based Therapies	miR-506 (targeting EMT regulators)	Preclinical evaluation for reducing metastasis	Preclinical	Minimal toxicity observed in preclinical models	(Sun et al. 2015)
Ribosome-Targeted Agents	CX-5461 (RNA Polymerase I inhibitor)	Targeting high ribosome biogenesis in cancer cells	Early-phase/preclinical studies	Potential for myelosuppression and off-target effects; under investigation	(Ivy et al. 2019)
Enzymatic Inhibitors	3-AP (ribonucleotide reductase inhibitor), inhibitors targeting StarD13	Overcoming platinum resistance; inhibiting metastasis mechanisms	Preclinical to early clinical trials	Varies; 3-AP associated with hematologic toxicity	(Ivy et al. 2019)
Gene Therapy	Ofranergene obadenovec (VB-111)	Platinum-resistant disease; experimental gene therapy	Evaluated in phase III trials (OVAL study)	Side effects similar to viral vector-based therapies; overall safety under review	(Arend et al. 2020)
Vaccines	Dendritic cell vaccines, Oregovomab	Stimulating immune response against tumor antigens	Limited clinical studies; early-phase trials	Generally mild; injection site reactions, low systemic toxicity	(Pietragalla et al. 2021)
Combination Therapies	PARP inhibitors + VEGF inhibitors (e.g., olaparib + bevacizumab), PARP inhibitors + immunotherapy, multi-agent immunotherapy	Enhancing efficacy and overcoming resistance in both frontline and recurrent settings	Multiple phase II/III trials ongoing	Cumulative toxicities (hematologic, vascular, immune-related), requiring careful management	(MRC Protein Phosphorylation and Ubiquitylation Unit n.d.)

1.3 PIN1 inhibitor

PIN1 is a specialized enzyme that catalyzes the *cis*–*trans* isomerization of phosphorylated serine/threonine-proline bonds, a key process that modulates the structure and function of many proteins. Its unique two-domain architecture features an N-terminal WW domain, which specifically binds to phosphorylated motifs, and a C-terminal PPIase domain responsible for catalyzing the isomerization reaction (Sudol 1996) as presented in Figure 3. This dual mechanism creates local structural changes that can significantly alter a protein's conformation, affecting stability, protein–protein interactions, and downstream signaling pathways involved in cell cycle regulation, oncogene activation, and tumor suppressor inactivation (Ranganathan et al. 1997). Chemically, PIN1 active site contains hydrophobic regions that anchor the proline residue and positively charged amino acids that interact with the phosphate group, thereby stabilizing the transition state during isomerization (Clackson and Wells 1995; Janin and Chotia 1990).

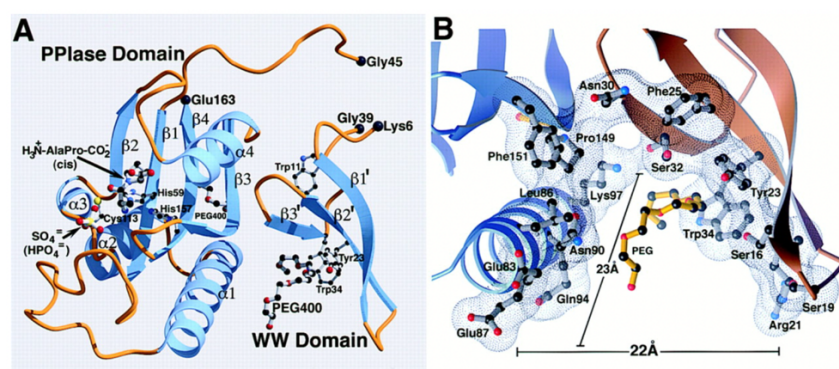


Figure 3 - Overall fold of Human PIN1. (A) Ribbon diagram of Pin1, apostrophes differentiate WW domain elements from those of the PPIase domain. Atoms are color-coded (red: O, blue: N, black: C, yellow: S) and HPO_4^{2-} indicates a phosphate substitute. (B) Ribbon and surface view of Pin1's interdomain cavity, showing the WW domain in orange and the PPIase domain in light blue. The dotted surface marks the largely hydrophobic, solvent-accessible region (Sudol 1996).

PIN1 facilitates the activation of over 40 oncogenes while simultaneously deactivating 20 tumor suppressors through its *cis*–*trans* isomerization mechanism. Its overexpression and heightened activity are linked to poor clinical outcomes, making PIN1 a compelling target for cancer therapy (La Montagna et al. 2013). Due to its central role in modulating phosphorylation-dependent signaling, PIN1 is often overexpressed in cancers and is a promising target for therapeutic intervention.

Over the past decade, a variety of PIN1 inhibitors have been developed using diverse strategies such as low-throughput PPIase screens, structural similarity analyses, mechanism-based high-throughput screening, substrate-mimicking designs, and binding assays (Dong et al. 2010). The earliest inhibitor, Juglone, is a naphthoquinone identified by its ability to inhibit both parvulin- and PIN1-type PPIases, effectively suppressing cell proliferation in cancers like hepatocellular carcinoma, prostate cancer, and glioblastoma, although its lack of specificity limits its use as a precise chemical probe (Guo et al. 2009). In contrast, all-*trans* retinoic acid

(ATRA) is an FDA-approved drug for acute promyelocytic leukemia that targets the PPlase catalytic domain of PIN1, thereby reducing cyclin D1 expression, blocking centrosome amplification, and inducing PIN1 degradation to inhibit its oncogenic functions, despite challenges with a short half-life and non-specificity (Wei et al. 2015). More recently, covalent fragment-based drug discovery led to the development of Sulfopin, a highly selective PIN1 inhibitor that targets the critical Cys113 residue. Sulfopin exhibits double-digit nanomolar potency and has been shown to engage PIN1 in cellular and in vivo models, leading to modest reductions in cancer cell viability, downregulation of Myc-dependent target genes, and significant tumor progression reduction in preclinical models of neuroblastoma and pancreatic ductal adenocarcinoma (Dubiella et al. 2021).

In 2019, a novel PIN1 inhibitor, VS10 (Figure 4), was discovered via consensus docking. It exhibits micromolar efficacy and holds promise as a therapeutic agent for tumors overexpressing PIN1. Treatment with VS10 in ovarian cancer cells induces proteasome-mediated degradation of PIN1, leading to reduced levels of downstream targets such as Ser473-pAkt, β -catenin, and cyclin D1 (Russo Spena et al. 2019).

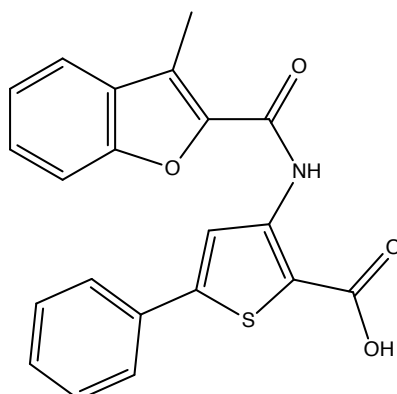


Figure 4 - Structure of PIN 1 inhibitor, VS10 (ChemDraw 16.0).

PIN1 is a unique peptidyl-prolyl cis-trans isomerase that exclusively catalyzes the cis-to-trans conversion of phosphorylated serine/threonine-proline motifs. This activity is critical for phosphorylation-dependent signaling, as proline-directed phosphatases only act on substrates in the trans conformation. It consists of two domains- a WW domain and a catalytic PPlase domain—joined by a flexible linker region (Pro37 to Pro52) as shown in the Figure 5 (Matena et al. 2018). The interaction between the WW and PPlase domains is highly dynamic in physiological conditions, differing significantly from the static orientation observed in crystal structures. VS10, a recently identified PIN1 inhibitor, demonstrates significant potential by interacting specifically with critical residues within the PIN1 active site. The ligand's carboxylic group forms essential ionic interactions with residues Arg69 and Lys63, while its thiophene moiety interacts specifically with the catalytic residue Cys113. Simultaneously, the phenyl ring of VS10 inserts deeply into the enzyme's

hydrophobic pocket defined predominantly by Leu122, Met130, Phe134, and His157. Furthermore, the 3-methylbenzofuran-2-carboxamide fragment establishes a stabilizing hydrogen bond with the hydroxyl group of Ser154, partially exposing it to solvent molecules. Thus, the unique structural and chemical properties of VS10 underscore its potential as a selective and effective PIN1 inhibitor, disrupting essential hydrophilic and hydrophobic interactions required for the enzyme's oncogenic functions (Russo Spena et al. 2019).

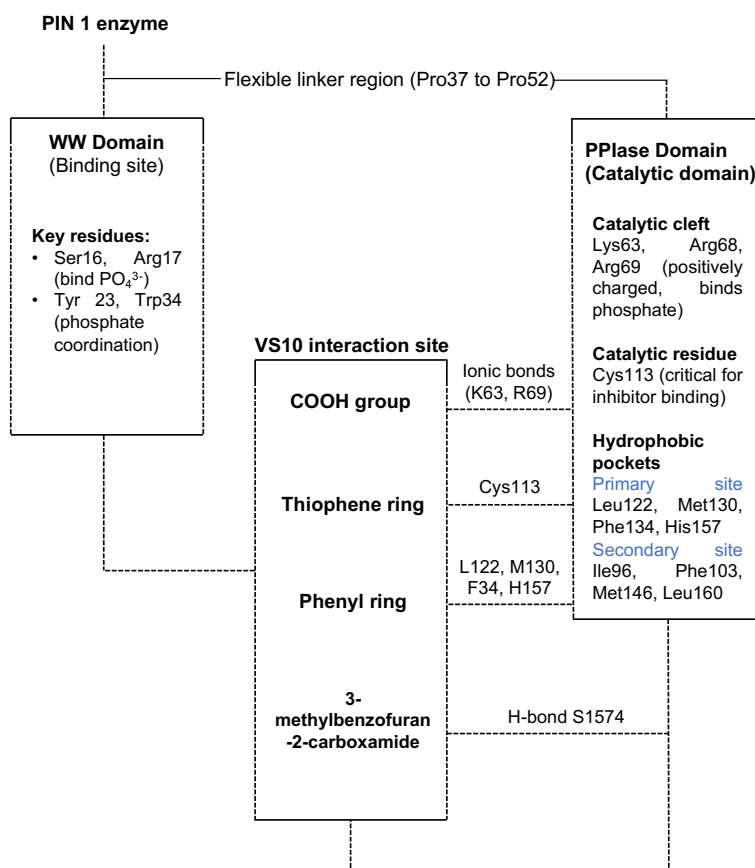


Figure 5 - Pin1 interaction sites with VS10. Flowchart simplifying the Pin1 structural features and VS10 binding mechanism (Matena et al. 2018; Russo Spena et al. 2019).

1.4 VS10 - BCS class 2 chemotherapeutic drug

VS10, known chemically as 3-(3-methyl-1-benzofuran-2-amido)-5-phenylthiophene-2-carboxylic acid, exhibits significantly low aqueous solubility (0.29 mg/mL at pH 7.4) despite possessing a high degree of lipophilicity (Log P = 5.52 and pH 7.4 log D = 2.17) analyzed via Chemicalize - Instant Cheminformatics Solutions. This characteristic places VS10 in Biopharmaceutics Classification system (BCS) Class 2 (Low solubility but high permeability). This intrinsic low solubility in water poses a substantial challenge to its bioavailability, as it limits the molecule's dissolution and subsequent absorption in the biological milieu. In contrast, its enhanced solubility in organic solvents such as DMSO indicates that the molecule's permeability is not the issue; rather, its solubilization in aqueous environments is inadequate.

To enhance the drug efficiency (Rizzolio et al. 2024) produced VS10/Pluronic F 127 crystals coated with albumin that resulted in 4 folds higher solubility than the VS10 alone. Consequently, the employment of a specialized carrier system, like liposomes, becomes imperative. Liposomal carriers can encapsulate the lipophilic drug, thereby improving its solubility and stability in aqueous media, facilitating targeted delivery, and ultimately enhancing its therapeutic performance.

1.5 Liposomal cellular uptake

Liposomal cellular uptake refers to the process by which liposomes with small, spherical vesicles composed of lipid bilayers are internalized into cells to deliver encapsulated therapeutic agents. The uptake efficiency critically determines the success of liposomal drug delivery systems, especially in cancer therapy. Typically, liposomal entry into cells occurs predominantly through energy-dependent endocytic mechanisms rather than passive diffusion. Endocytosis is initiated when liposomes interact with the cellular plasma membrane, either through nonspecific interactions or, more commonly, via receptor-mediated pathways that exploit specific receptors expressed on the surface of cancer cells (Torchilin 2007). Following internalization, liposomes are initially encapsulated within membrane-bound vesicles called endosomes. Early endosomes, characterized by slightly acidic pH (~6.0–6.5), mature progressively into late endosomes and ultimately fuse with lysosomes, which possess an even lower pH (~4.5–5.0). Within these acidic environments, liposomes lacking specialized escape mechanisms may become degraded along with their encapsulated drugs, significantly reducing therapeutic efficacy. Therefore, achieving efficient endosomal escape is crucial for effective intracellular drug delivery. To promote successful endosomal escape, liposomes often incorporate fusogenic lipids (e.g., distearylphosphatidylethanolamine, DSPE) or specialized peptides that undergo structural transformation under acidic conditions, disrupting the endosomal membrane and allowing liposomal contents to be released directly into the cytosol (Danhier, Feron, and Pr  at 2010). Liposomes typically range from 50 nm to 400 nm, with smaller liposomes (<200 nm) generally exhibiting superior cellular internalization. Surface charge also affects the interaction between liposomes and cell membranes: neutral or slightly negatively charged liposomes minimize nonspecific interactions with serum proteins and immune cells, thus extending circulation times and improving selective uptake (Wang and Thanou 2010). Conversely, liposomes with highly positive charges can induce nonspecific interactions and rapid clearance, though moderate positive charges may sometimes enhance cellular uptake in controlled scenarios. PEGylation is widely used to extend the circulation time of liposomes in the bloodstream by preventing their rapid clearance by the immune system. However, this beneficial modification can inadvertently create challenges, as the dense, hydrophilic PEG layer on the liposome surface may act as a barrier that interferes with efficient

intracellular uptake or subsequent drug release within cells (Wang and Thanou 2010). Specifically, after internalization through endocytosis, liposomes with persistent PEG coatings may become trapped inside endosomes, preventing their escape into the cytoplasm. To address this limitation, researchers have developed advanced liposomal formulations that exploit the subtle pH differences between healthy tissues and pathological or intracellular environments (Zhao, Zhuang, and Qi 2011). These pH-responsive liposomes incorporate specially designed cleavable linkages between the PEG chains (hydrophilic moieties) and lipid anchors (hydrophobic moieties). For example, the PEG linked-lipid, DSPE-PEG 2000 which was used in the Doxil preparation (Perche and Torchilin 2013). Cholesterol plays a crucial role in enhancing the cellular uptake by increasing liposomal membrane stability and optimizing membrane fluidity in plasma. Cholesterol plays a crucial role in enhancing the structural stability of liposomes in plasma. Its presence diminishes liposomal interactions with various proteins, reducing susceptibility to phospholipases, inhibiting the loss of phospholipids to high-density lipoproteins, altering membrane enzyme activities, and decreasing both macrophage-mediated digestion and unwanted fusion events. These effects collectively help regulate blood clearance and tissue distribution of liposomes after intravenous administration. Specifically, incorporating cholesterol can lessen reticuloendothelial uptake by minimizing liposome–protein interactions, thereby affecting tissue uptake (D’Avanzo et al. 2011).

2. Aim of Work

The main goal of this research is to design and optimize a microfluidics-based liposomal carrier system for VS10, a chemotherapeutic agent, to improve its bioavailability. By systematically varying the Flow Rate Ratio (FRR) and the Total Flow Rate (TFR), along with exploring different lipid compositions, the study seeks to identify an optimal microfluidic process that yields VS10 liposomes with the most favorable physicochemical properties. Comprehensive characterization includes measuring particle size, polydispersity index (PDI), and zeta potential to ensure colloidal stability. Transmission electron microscopy (TEM) is used to confirm liposome morphology, while drug encapsulation efficiency, drug loading capacity, and lipid quantification verify the integrity and capacity of the liposomal systems. In vitro drug release studies at pH 7.4 provide insights into the release kinetics and stability of VS10 within the liposomes. Finally, evaluating the cytotoxic potential on ovarian cancer cell lines via IC₅₀ measurements compares free VS10 against VS10-loaded liposomes, confirming whether the liposomal formulation offers superior therapeutic efficacy and stability over the free drug.

3. Material and Methods

3.1 Materials

The VS10, 3-(3-methyl-1-benzofuran-2-amido)-5-phenylthiophene-2-carboxylic acid, (MW: 377.41 g/mol) compound was produced by Enamine, Kiev, Ukraine. Bradford dye reagent (Cat. No. 500–0006) was procured from Bio-Rad, Hercules, CA, USA. Lipids were bought from Avanti Polar Lipids: HSPC (Hydro Soy, 840058); Cholesterol (CHO) (700000); 18:0 DSPE-PEG2000 (880120). Materials bought from Merck: Dulbecco's Phosphate Buffered Saline (DPBS), (D1408); AM (A8423); deuterated chloroform (151,823); penicillin–streptomycin (P4333). Absolute ethanol (V001229) and DMSO were bought from Sigma Aldrich. OVCAR5, SKOV-3, and Kuramochi cell lines, representing ovarian cancer, were generously provided by Dr. Gustavo Baldassarre at the National Cancer Institute CRO-Aviano. Water was purified using a Milli-Q® (Millipak® 0.22 µm) system.

3.2 Intrinsic solubility analysis of VS10

Solubility of VS10 was estimated in ethanol and water, procedure was in accordance with Higuchi and Connors, an excess amount of drug (20 mg) was added in 1 mL of the solvents (ethanol and water), the solution was vortexed, sonicated at 37 °C for 20 mins and was kept for 24 hours in the thermal shaker at 37 °C at 100 rpm to attain the equilibrium between the solvents. The measurements were recorded in triplicates. The samples were centrifuged at 37°C to maintain the equilibrium at the speed of 5000 g for 30 mins. To estimate the aqueous solubility of VS10, the pellet obtained after the centrifuge was estimated by resuspending in DMSO, the amount quantified was subtracted with the initial amount to know the quantity in the aqueous phase (pH 7.4). Meanwhile supernatant was collected of the vs10/ethanol and both of the drug/solvent solubilities were estimated using UV/Vis spectroscopy at 302 nm.

3.3 Microfluidics Synthesis

To produce both the empty and quercetin loaded liposomes was applied a microfluidics setup composed by commercially available components: Microfluidics apparatus (Sunshine, Unchained labs, Milan, Italy) constitute of pressurized fluid bottles, 2 quad pumps (Organic and aqueous), automated sample valve and collector. The instrument has a microfluidics chip holder named as sunny rail where the microfluidic chip (Sunnies) is combined with the loop system (10 mL loop) present in the automated sample valve. In the experiment, glass microfluidics chip with T and X junctions with 190 µm etch depth was used. The chip is a laminar flow reactor exhibiting the pockets of chaotic advection when used under optimal conditions. The microfluidic device was controlled by the Sunny Suite Software which allowed to create protocols and automated experiments.

3.3.1 Blank and VS10 loaded liposome preparation

Blank and Vs10 loaded liposomes were prepared using a lipid mixture of HSPC: CHO: DSPE-PEG (55:40:5) molar ratios corresponding to 10 mM that were chosen in accordance with (Niu, Guoqin, Brian Cogburn, and Jeffrey Hughes.,2010).

Initially, the lipid mixture (HSPC: CHO: DSPE-PEG) was dissolved in ethanol (5 mL) to maintain the desired molar ratio, and the solution was heated to 60°C to ensure that the lipids were above its phase transition temperature, thereby achieving a homogeneous, fluid state. For VS10 loaded formulations, the drug (1mg/mL) was incorporated into the ethanol: DMSO (1:1) solution (5 mL) along with the lipids at a similar concentration as blank, and quantities were adjusted according to the flow rate ratios (FRRs) required for subsequent microfluidic processing. Liposome formation was conducted using 190 XT chip provided by the Sunshine, Unchained Labs. FRR conditions of 3 and 5, with total flow rates (TFRs) varied between 4 and 12 mL/min, while a 10 mM DPBS aqueous phase was consistently used.

In the current experiments, the effective lipid concentration in the final liposomes is dictated primarily by the FRR rather than the TFR. For example, to obtain the 10 mM as the lipid concentration in the final formulation, the lipids dissolved in ethanol solution should be 4 times more as an initial solution when preparing for FRR 3 and similarly, using a FRR of 5, the dilution factor would be 6, requiring a 60 mM initial solution to reach 10 mM in the final formulation simplified in Table 2.

Table 2 - Required initial and final lipid molarities (mM) for maintaining a 5.5:4:0.5 molar ratio of HSPC: CHO: DSPE-PEG at target final total lipid concentration at 10 mM using an FRR 3 and 5.

HSPC: CHO: DSPE-PEG			
Flow rate ratio (FRR)	Initial lipid Molarity (mM)	Final lipid Molarity (mM)	Lipid Concentration (mg/mL)
3 (<i>dilution factor 4</i>)	40 (22:16:2)	10 (5.5:4:0.5)	7.24
5 (<i>dilution factor 6</i>)	60 (33:24:3)	10 (5.5:4:0.5)	7.26

The collected samples were dialyzed overnight against DPBS (10 mM) using the dialysis tubing cellulose membrane (14 kDa MWCO; Merck) to eliminate unencapsulated molecules and residual ethanol, thereby preventing lipid interdigitation within the bilayer and preserving the structural integrity of the liposomes.

3.4 Dynamic Light Scattering (DLS)

The particle size, polydispersity index (PDI) and zeta potential were examined by Zetasizer Nano particle analyser (Malvern Panalytical, Malvern, UK). The size was measured at 25°C, equilibrating time of 60 seconds. The samples were diluted from 1:20 to 1:100 to optimize the

desired dilution for the estimation. DLS was conducted on all samples prepared under the varied conditions. Only those samples meeting the optimal criteria were selected for further analysis, while the remainder were excluded. VS10 liposome were stored at 4°C for one month and the stability was evaluated to estimate the deterioration of the sample dispersed in DPBS for particle size and PDI.

3.5 Transmission Electron Microscopy (TEM)

A 25 µL drop of the solution was placed onto a 400-mesh holey film grid. Following a 2-minute staining with 1% uranyl acetate, the samples were examined using a FEI Tecnai G2 transmission electron microscope (Hillsboro, Oregon, US) operating at 100 kV. Images were captured with a Veleta digital camera (Olympus Soft Imaging System) and processed using ImageJ software.

3.6 Phospholipid Quantification using Stewart assay

Lipid quantification was performed using the Stewart assay. The ferro thiocyanate reagent was prepared following the protocol (Prima, Librizzi, and Carrotta 2020) where 27.03 g of ferric chloride hexahydrate (Sigma Aldrich) and 30.4 g of ammonium thiocyanate (Sigma Aldrich) were dissolved in double distilled water and brought to a final volume of 1 L; the resulting solution remains stable at room temperature for several months. In glass tubes, 1.5 mL of chloroform and 1.5 mL of the prepared Stewart reagent were added, and each condition was prepared in triplicate. For the calibration curve, 20 µL of a blank lipid standard ranging from 2 to 10 mM were added in the glass tubes and were prepared in triplicates. The assay, which relies on the formation of a colored complex between phospholipids and ammonium ferrothiocyanate in an organic solvent, was measured at 485 nm. After vortexing, the samples were centrifuged at 5000 rpm for 15 minutes at 20°C, and the supernatant was collected for phospholipid calibration curve using UV spectroscopy. To quantify the lipid concentration, 20 µL of the blank liposome was similarly added in another glass tube containing the chloroform and Stewart reagent which was further vortexed and centrifuged using the similar conditions and supernatant was estimated.

3.7 Encapsulation efficiency (EE %) and Drug loading (DL %)

The quercetin liposomal samples (500 µL) were added in absolute ethanol (500 µL), vortexed and were bath sonicated at 50°C for 20 mins. The samples were collected and centrifuged at 17,000 g, 4°C for two hours with Micro Star 17R (Avantor, Radnor, Pennsylvania, USA). The supernatant was collected and diluted 1:10 with ethanol and concentration of VS10 was estimated at 302 nm using Cary 100 UV-Vis Spectrophotometer, Agilent.

3.8 In Vitro drug release

In vitro release tests were conducted on VS10 liposomes in a physiological pH 7.4 medium (10 mM), prepared in triplicate. To maintain the solubility of VS10, 10% absolute ethanol was added to each medium. The VS10 liposomes were placed inside a dialysis tubing of MWCO 5000 Da, Spectra/POR® 3 purchased from VWR, which was then immersed in a beaker containing 50 mL of release medium. The experiment was carried out at 37°C under continuous stirring (100 rpm).

At predefined time intervals (2, 4, 6, 24, and 48 hours), 20 µL samples were collected from inside the dialysis membrane, diluted 1:50, and analyzed using a Cary 100 UV-Vis Spectrophotometer at 302 nm. Before absorbance measurements, the baseline was established using the release medium containing 10% ethanol. The drug concentration was determined by correlating the absorbance values with the calibration curve of VS10 in ethanol, and the drug release profile was subsequently calculated.

3.9 Cytotoxicity analysis

The half-maximal inhibitory concentration (IC₅₀) was evaluated on the Kuramochi, OVCAR5 and SKOV-3 ovarian cancer cell lines. Cells were seeded in 96-well plates at a density of 1,500 cells per well. On the following day, the cells were treated with serial dilutions in culture media. Cisplatin, used as a control, was tested with serial 1:10 dilutions starting at 100 µM, while the other compounds were tested with serial 1:2 dilutions starting at 200 µM. After the specified incubation period, cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay on a Synergy H1 plate reader, and IC₅₀ values were calculated using GraphPad Prism software.

4. Results and Discussion

4.1 Solubility analysis of VS10

VS10 (3-(3-methyl-1-benzofuran-2-amido)-5-phenylthiophene-2-carboxylic acid) demonstrates pronounced hydrophobic characteristics, as evidenced by its low aqueous solubility of approximately 0.05 mg/mL and higher solubility in ethanol at about 0.72 mg/mL. This solubility behavior can be attributed to the extensive aromatic framework of the molecule, which can be seen in the structure as it contains: a benzofuran ring, a thiophene ring, and a phenyl substituent. These π -conjugated systems greatly increase the lipophilic surface area, reducing the overall polarity of compound. While the amido and carboxylic acid functional groups can form hydrogen bonds, they do not sufficiently offset the hydrophobic domain created by aromatic rings. The ability of VS10 to dissolve under sonication and elevated temperatures (up to 50 °C) highlights its permeability, but it also underscores the relative lack of intrinsic hydrophilicity. Furthermore, these experimental findings are in line with Chemicalize - Instant Cheminformatics Solutions based computational predictions.

Table 3 - Solubility of VS10 in the selected organic solvent (Ethanol) and aqueous solvent pH 7.4 estimated using U/VIS Spectroscopy. The values are expressed in g/L (SD).

Solubility of VS10 $\pm$ SD (g/L)	
Ethanol	Water
0.73 $\pm$ 0.17	0.05 $\pm$ 0.06

4.2 Microfluidics optimization of VS10 liposomes

The production of blank and VS10 loaded liposomes was studied considering the influence over samples with different flow rate ratio (FRR 3 and 5), total flow rate ratios (2, 6, 8, 10, 12 mL/min), at lipid concentration of 10 mM. When preparing liposomes via microfluidic mixing, several interdependent parameters—flow rate ratio (FRR), total flow rate (TFR), total lipid concentration—govern the nucleation and growth of lipid vesicles, ultimately affecting liposome size, polydispersity index (PDI), and stability.

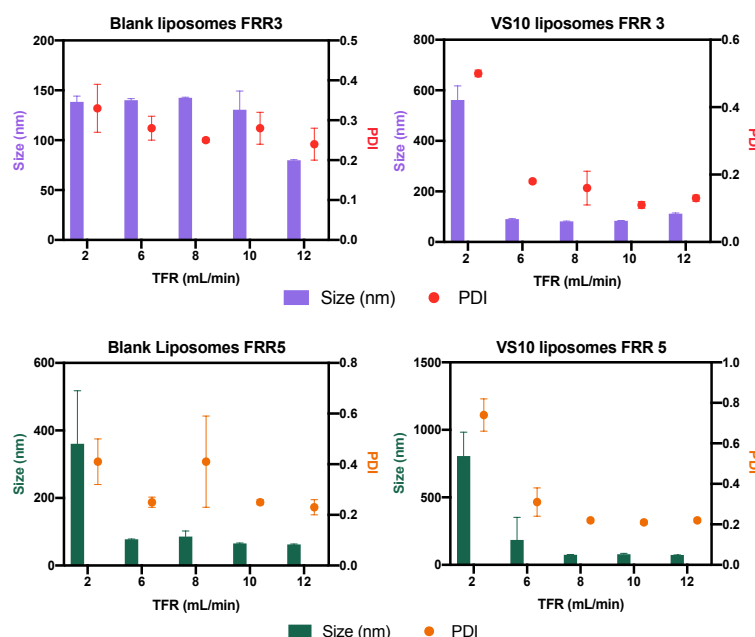


Figure 6 - Optimization of microfluidics parameters. Particle size and PDI of Blank and VS10 liposomes obtained using FRR 3 and 5 at TFR 2-12 mL/min using the Laminar-chaotic (190 XT) chip.

The obtained results in Figure 6 clearly demonstrate the impact of total flow rate (TFR) and flow rate ratio (FRR) on liposome particle size and polydispersity index (PDI) when prepared via microfluidics. At low TFR (2 mL/min), poor mixing and slow diffusion of the lipids into the aqueous environment result in large, heterogeneous liposomes (561.8 nm, PDI 0.5 at FRR 3; 806.5 nm, PDI 0.74 at FRR 5), indicating inadequate lipid self-assembly under these conditions (Jahn et al. 2010). Increasing TFR 6–12 mL/min significantly enhances mixing efficiency and accelerates lipid nucleation, markedly reducing liposome size and improving uniformity. The mixing of lipids which are suspended in 25 % ethanol at FRR 3, liposomes ranged from 81.55–111.9 nm with PDIs between 0.11–0.18, while at FRR 5, even smaller and highly uniform liposomes (73.1–78.8 nm, PDI 0.21–0.22) were consistently obtained. These observations align with literature findings indicating optimal liposome production at intermediate-to-high TFR and higher FRR, which balances rapid solvent dilution and effective lipid assembly, thus confirming the suitability of TFR $\geq$ 8 mL/min combined with higher FRR 5 for achieving reproducible and homogeneous liposomal formulations (Kastner et al. 2014). From the extensive set of experiments conducted, six specific liposomal formulations emerged as particularly promising based on their therapeutic potential. The results obtained by the FRR 3 and 5 at the TFR 8, 10, and 12 mL/min were selected then re-created to verify reproducibility, and further characterizations were carried out to confirm their stability and efficacy.

4.3 VS10 liposomes characterization

4.3.1 Dynamic Light Scattering (DLS)

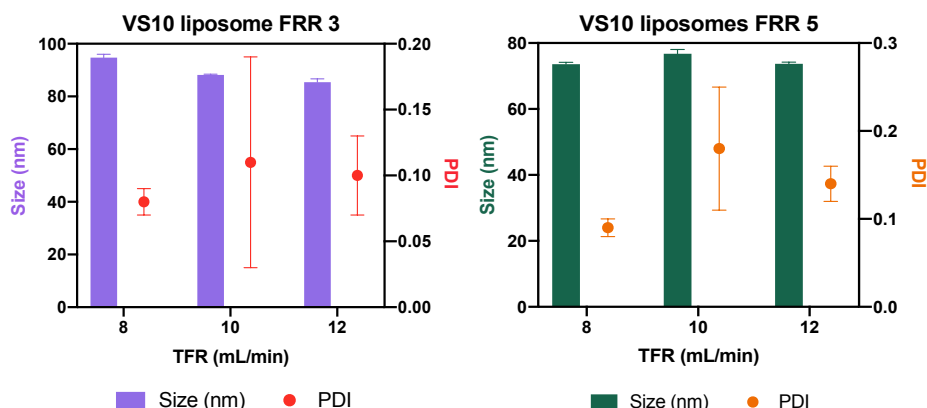


Figure 7 - Comparison of the selected liposomes. Average particle size (bars) and polydispersity index (symbols) of VS10 liposomes under two different conditions of FRR 3 and 5 at TFR of 8, 10, 12 mL/min.

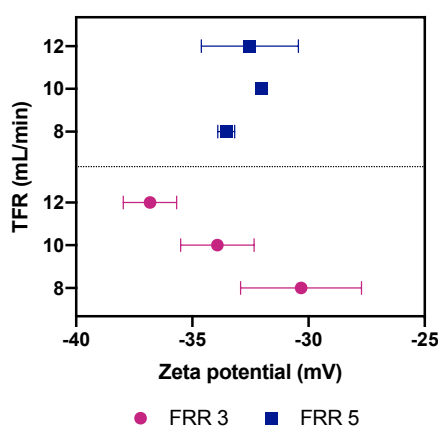


Figure 8 - Zeta potential analysis. VS10 liposomes prepared via microfluidics at three total flow rates (TFRs) of 8, 10, and 12 mL/min under two distinct flow rate ratios (FRRs): FRR 3 (pink circles) and FRR 5 (blue squares). Each data point represents the mean $\pm$ standard deviation, indicating the stability and uniformity of the liposomal suspensions. Higher absolute zeta potential values (closer to -30 mV or beyond) suggest enhanced electrostatic repulsion among particles, reducing the likelihood of aggregation and contributing to improved formulation stability.

The reproducibility studies using optimized parameters (FRR 3 and FRR 5 at TFR 8, 10, and 12 mL/min) confirm that particle size and polydispersity index (PDI) remain consistently favorable, reflecting improved control over liposome formation presented in Figure 7. The particle size obtained were prepared at 1:20, 1:50, 1:100 times dilution with DPBS pH 7.4 10 mM and among which the sizes at 1:50 comparatively showed better results. For FRR 3, liposome sizes ranged from approximately 85.4 nm to 94.7 nm with consistently low PDIs (0.08–0.11), demonstrating effective and reproducible mixing at varied TFR. For FRR 5, even smaller liposomes (73.6–76.7 nm) were produced with similarly low PDIs (0.09–0.18), indicating a more rapid solvent dilution and nucleation process due to higher aqueous-to-organic phase ratio. These findings are in line with established literature indicating that higher FRR ≥ 5 often yields smaller liposomes due to rapid lipid precipitation, while TFR increases from 8 mL/min to 12 mL/min efficient mixing and homogeneous particle formation occurs

(Kastner et al. 2014). The selected aqueous medium has been shown to influence as well on both the size and distribution of liposomes, with lower ionic strength (8 mM) conditions producing smaller, more uniformly dispersed vesicles. Liposomes also poses the stearic stability due to the PEGylation which prevents aggregation hence reduction in the particle size. Slight variations in particle size and PDI between replicate preparations can be attributed to minor fluctuations inherent to microfluidic systems and while sample preparation but are well within acceptable ranges, thus supporting the reliability and robustness of the optimized parameters.

Zeta potential is an indicator of the electrostatic repulsion among liposomes, and it varies based on surrounding media/solvent. Consequently, it serves as a key measure of a liposomal physical stability. As shown in Figure 8, liposomes freshly prepared with HSPC: CHO: DSPE-PEG 2000 loaded with VS10 yielded relatively high absolute zeta potential values (-30 mV to -36 mV) under all microfluidics conditions. Zeta potentials of ± 30 mV or higher generally signify a stable suspension, attributed to reduced particle aggregation. This stability arises from hydrogen bonding interactions among phospholipids, cholesterol, and the hydrophilic PEG segments, which create a highly electronegative surface and thus diminish the likelihood of vesicle aggregation.

4.3.2 Stability over time

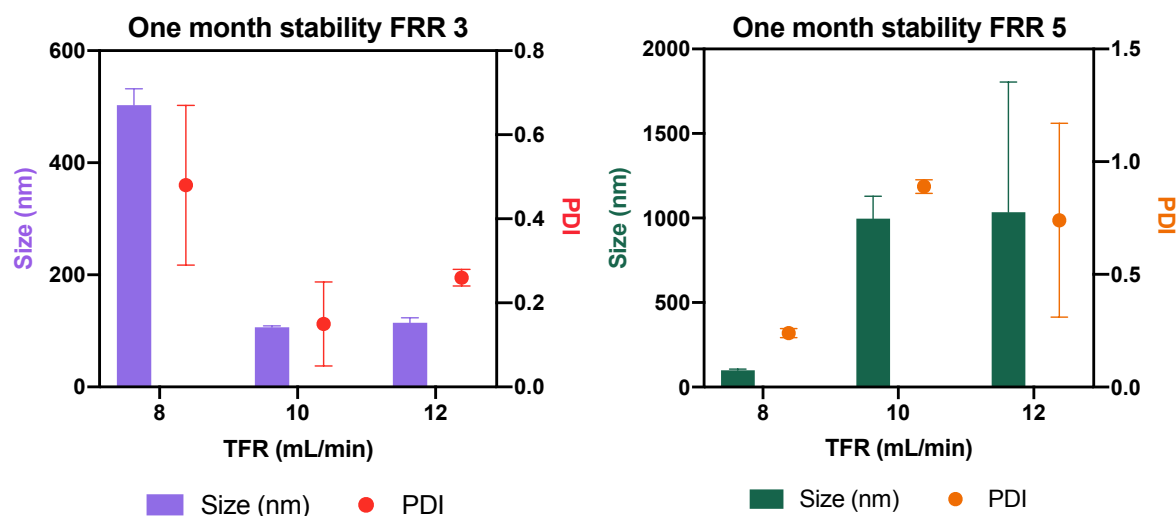


Figure 9 - Stability over one month. One-month stability evaluation of VS10 liposomes formulated at different flow rate ratios (FRRs) and total flow rates (TFRs). Liposomes prepared at FRR 3, TFR 8 mL/min showed a dramatic size increase from 95 nm to 502 nm with high PDI (0.48). By contrast, liposomes fabricated at FRR 3 and TFR 10–12 mL/min exhibited only a modest size rise (85–110 nm), demonstrating more stable formulations under identical storage conditions. VS10 liposomes prepared at FRR 5 with TFR 8, 10 and 12 mL/min demonstrating huge increase in particle size and PDI due to the aggregation during the storage conditions.

A one-month stability assessment of VS10 liposomes revealed that formulations prepared with an FRR of 3 and a TFR of 8 mL/min experienced a pronounced increase in particle size, rising from 95 nm to 502 nm, accompanied by a high PDI of 0.48. This indicates substantial heterogeneity and instability. The TEM images (Figure 10) support these findings, showing varied particle sizes and broken vesicles on some areas of the grid. In contrast, liposomes fabricated at the same FRR but with TFRs of 10 and 12 mL/min displayed only a modest size increase (85–110 nm). Conversely, VS10 liposomes prepared using FRR 5, showed massive rise in the sizes from 73 nm to 1 μ m with PDI of 0.89 indicating aggregation of the liposomes during the period of one month storage (Figure 9).

Several factors likely contributed to the observed size expansion over one month. First, osmotic stress at the storage temperature (4 °C) can weaken the liposomal membrane, causing vesicles to swell or fuse. Second, phospholipids may undergo phase transitions at this low temperature, which can alter membrane fluidity and compromise the vesicles' structural integrity. Third, gradual leakage of the encapsulated VS10 could change the internal environment of the liposomes, further affecting their stability. Finally, diminished interactions between cholesterol and phospholipids over time may reduce the overall rigidity and stability of the bilayer, promoting liposome enlargement and heterogeneity.

4.3.3 Transmission Electron Microscopy (TEM)

TEM images of the liposomal formulations reveal distinct morphological differences between the samples prepared at FRR 3 and 5 using TFR 8-12 mL/min. In few sets of images, the liposomes appear predominantly as smaller, well-defined spherical vesicles with visible bilayer structures. Their relatively uniform shape and diameter, along with only slight size variations, agree with the dynamic light scattering (DLS) results indicating a narrower distribution as observed in Figure 8.

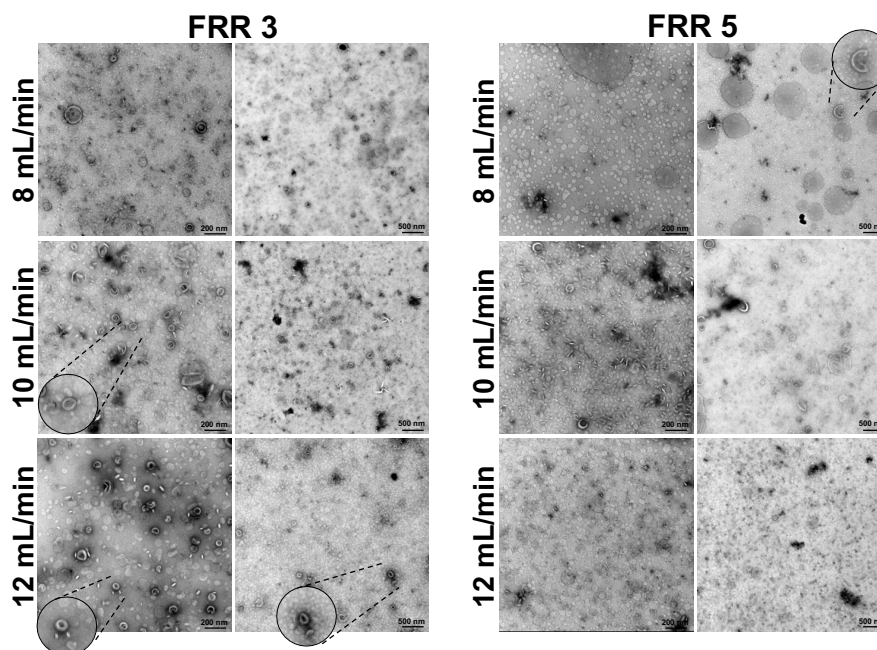


Figure 10 - TEM analysis. On left TEM images representing the VS10 liposomes at FRR 3 and on right VS10 liposomes prepared at FRR 5 using TFR 8, 10, 12 mL/min in each case.

Transmission electron microscopy (TEM) images were acquired at 200 nm and 500 nm scales for liposomes prepared under flow rate ratios (FRR) of 3 and 5, at total flow rates (TFR) of 8, 10, and 12 mL/min. In both FRR 3 and FRR 5 at 8 mL/min, liposomes appeared spherical but exhibited a wide range of particle sizes (from larger to smaller vesicles). At FRR 5 with 8 mL/min and 500 nm scale, a broken liposome was observed, likely caused by bilayer stress. This happens when the lipid bonds are already in a loosened state as the sample procedure and imaging is performed by cryo-TEM, a technique designed to preserve the native, hydrated state of the liposomes. For TFR 10 mL/min with both FRR 3 and FRR 5, liposomes again displayed spherical and also elongated shapes. Small size of the vesicles is also confirmed by the DLS analysis obtained in the range of 70-80 nm meanwhile, TFR 12 mL/min, FRR 3 yielded a unimodal size distribution with all liposomes maintaining a spherical shape. In contrast, FRR 5 at 12 mL/min also produced spherical vesicles but with occasional broken liposomes. As observed, the liposomes obtained using FRR 3 with TFR 12 mL/min showed a balance between the lipid-aqueous phase ratio that did not produce the shear stress thus stabilising the liposomes.

4.3.4 Encapsulation efficiency (EE %), Phospholipid quantification and Drug loading (DL %)

Table 4 - Encapsulation efficiency. VS10 liposomes prepared using microfluidics at FRR 3 and 5 using TFR of 8, 10, 12 mL/min where VS10 feed was set at 0.287 mg/mL (FRR 3) and 0.267 mg/mL (FRR 5).

FRR	Encapsulation efficiency % at TFR (mL/min)		
	8	10	12
3	66 ± 2	69 ± 2	55 ± 13
5	58 ± 2	55 ± 1	49 ± 1

The encapsulation efficiency is estimated using the equation:

$$EE \% = \frac{\text{Amount of VS10 observed}}{\text{Initial amount of VS10 added}} \times 100$$

Recent studies have shown that incorporating lipids with shorter hydrocarbon chains (e.g., 12:0 PC to 14:0 PC) in liposomal formulations results in reduced drug encapsulation efficiency and faster drug release (Anderson and Omri 2004). In contrast, using phospholipids with longer acyl chains leads to a higher drug loading capacity, likely due to the increased bilayer area provided by the extended lipid chains. HSPC (Hydro Soy) is composed of fatty acid chains that are around 18 carbons long. Longer the acyl chain, better the stability and hence the encapsulation and loading capacity as mentioned in Table 4. In the preparation 18:0 DSPE-PEG 2000 was used as well that contributes in spatial stability of the liposomes meanwhile PEG chains providing steric stability and minimizing drug leakage over time and storage. Cholesterol concentration at molar ratio of 40 enhanced the stability of the liposomes, maintaining the VS10 loading, minimizing the drug leakage thus improving the encapsulation. The theoretical loading capacity was determined to be 12.6% for FRR 3 and 11.6% for FRR 5, based on a theoretical phospholipid concentration of 6 mM yielding 79.3 % of phospholipids in the liposomes. Drug loading was determined based on the total lipid concentration. Prior to this calculation, the phospholipid content of the vesicles was quantified using the Stewart assay to accurately measure the lipids that formed the vesicular structures. A calibration curve was prepared, yielding a linear equation ($y = 44.4 x$) with a regression coefficient of 0.9978, as illustrated in Figure 11. The slope ($\epsilon = 45.259$) was then used to calculate the phospholipid content in the liposomes where the lipid concentration of 5.2 mM was obtained.

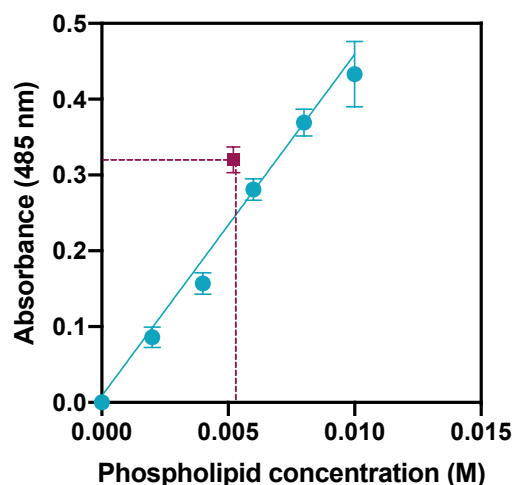


Figure 11 - Phospholipid Calibration Curve. The graph depicts the linear correlation between absorbance at 485 nm and lipid concentration (M) shown by teal colour trendline. The linear equation obtained was $y = 44.4x + 0.0031$ with $R^2=0.9978$. Dark red square points represent phospholipid quantification of FRR 3 and 5 at 5.2 mM

The drug loading was hence calculated using:

$$DL \% = \frac{\text{Molar concentration of VS10}}{\text{Molar concentration of lipids}} \times 100$$

Table 5 - Drug loading. VS10 liposomes prepared using microfluidics at FRR 3 and 5 using TFR of 8, 10, 12 mL/min where VS10 feed was set at 0.287 mg/mL (FRR 3) and 0.267 mg/mL (FRR 5).

FRR	Drug loading % at TFR (mL/min)		
	8	10	12
3	9.7 ± 0.2	10 ± 0.2	8 ± 1.89
5	7.8 ± 0.2	7.5 ± 0.1	6.7 ± 0.1

4.3.5 In Vitro drug release

Depending on the results obtained from DLS, TEM, EE % and DL%, VS10 liposome sample prepared at FRR 3 using TFR 10 mL/min was chosen further in-vitro drug release analysis at physiological pH conditions in Figure 12. The release was expected to have a sustained drug release profile because of HSPC lipids with longer fatty acid chains have larger polar head groups, stronger lipid-chain cohesion forces, and greater steric effects, which also make them more resistant to hydrolysis. In this study, liposomes containing these lipids exhibited a more prolonged drug release up to 48 hours.

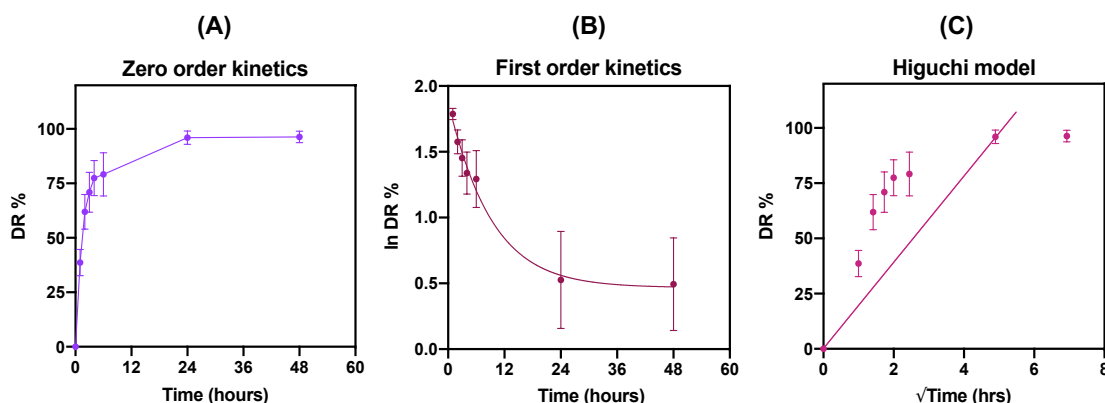


Figure 12 - In vitro release profiles of the VS10 liposomes at pH 7.4 fitted to three kinetic models. (A) Zero-order plot (Drug Release vs. Time) with a slope of 2.76%/hr ($R^2 = 0.546$). (B) First-order plot [$\ln(100-DR)$] vs. Time) with a slope of -0.05 hr^{-1} ($R^2 = 0.989$). (C) Higuchi plot (Drug Release vs. $\sqrt{\text{Time}}$) with a slope of 19.72 ($R^2 = 0.832$). The high R^2 in the first-order plot suggests that the release rate is primarily governed by the concentration of drug remaining in the liposomes.

At physiological pH (7.4), the liposomal formulation exhibits a clear biphasic release profile, characterized by a rapid burst phase (0–2 hours) that increases drug release from 0% to about 62%, followed by a slower, sustained phase (2–48 hours) reaching near-complete release (96%) by 24–48 hours. This early burst likely arises from drug loosely associated with or near the liposome surface, while the subsequent slower release is governed by diffusion from the liposomal core. Variability in intermediate time points suggests a complex release mechanism that can be explored via kinetic modelling. A single-phase model might capture the overall release plateau (96–97%) with a rate constant of 0.5 h^{-1} but overlooks the early burst, whereas a biphasic model (with fast and slow components) more accurately reflects both the initial surge and the later sustained diffusion.

Table 6 - Representation of the slope and linear regression derived from the three kinetic model of drug delivery system, Zero Order, First order, and Higuchi.

Model	x-axis	y-axis	Slope	R^2
Zero order	Time (hr)	DR %	2.76 %/hr	0.546
First order	Time (hr)	$\ln(100-DR)$	-0.05/hr	0.989
Higuchi	$\sqrt{\text{Time}}$	DR %	19.72	0.832

Standard kinetic models can also be applied in these forms presented in Table 6 (Paul 2011)

a) Zero order

$$DR \% = Slope \times time + intercept$$

where slope represents the constant release rate.

b) First order

$$\ln(100 - DR \%) = Slope \times time + intercept$$

where the transformation converts the exponential decay into a linear trend. The fitted line negative slope corresponds to the rate at which the unreleased fraction decreases.

c) Higuchi model

$$DR \% = k\sqrt{t} + intercept$$

Based on these three classical kinetic models, first-order fits statistically better and the results are in accordance with (Smits et al. 2019) where the first-order kinetics is commonly observed in liposomal drug delivery systems. Under this model, the release rate is proportional to the amount of drug remaining in the liposome. Practically, this translates into a rapid initial release—which can be desirable for a prompt therapeutic effect—followed by a slower release phase as the internal drug reservoir depletes.

4.3.6 Cytotoxicity analysis

Table 7 - Cytotoxicity profile in ovarian cell lines. IC₅₀ ± SD were recorded of free VS10 and VS10 liposomes on OVCAR5, SKOV3 and KURAMOCHI calculated using GraphPad Software.

	OVCAR5	SKOV3	KURAMOCHI
VS10-free	40.1 ± 2.6	38.4 ± 3.00	52.4 ± 5.4
VS10-liposomes	44.0 ± 5.8	38.6 ± 4.2	66.2 ± 2.2

The study evaluated the cytotoxic effects of both free VS10 and its PEGylated liposomal formulation that were prepared at a FRR 3 and a TFR of 10 mL/min on OVCAR5, SKOV3, and Kuramochi ovarian cancer cell lines. Both formulations effectively inhibited cell viability; however, the PEGylated VS10 liposomes demonstrated a notably lower IC₅₀ compared to the free drug, as shown in Table 7. This lower IC₅₀ indicates that the liposomal form is more potent, likely due to enhanced drug delivery mechanisms. The PEGylation process improves the liposomes' stability, prolongs their circulation time, and facilitates better tumor targeting, which collectively contribute to the increased anticancer efficacy observed in these ovarian cell lines.

5. Conclusion

The microfluidic optimization of blank and VS10-loaded liposomes has demonstrated that both total flow rate (TFR) and flow rate ratio (FRR) play critical roles in determining the quality of the liposomal formulations. At lower TFR (2 mL/min), inadequate mixing led to larger, more heterogeneous vesicles, whereas increasing TFR (8–12 mL/min) significantly enhanced mixing efficiency and accelerated lipid nucleation. This resulted in markedly smaller liposomes with low polydispersity indices, particularly when using a higher FRR 5, which favors rapid solvent dilution and lipid self-assembly. The reproducibility studies confirmed that optimized parameters (FRR 3 and 5 at TFR 8, 10, and 12 mL/min) yield consistent and robust liposomal characteristics, corroborated by both dynamic light scattering and transmission electron microscopy. These findings not only align with literature reports but also paved the way for the selection of six promising formulations for further characterization in terms of stability, encapsulation efficiency (60-70%), and drug loading (8-10%). The biphasic release of the liposomes was fitted according to the FIRST order kinetic release where the release rate is proportional to the amount present in the liposomes and thus providing the sustained release profile. The cytotoxicity effects of the VS10 and VS10 liposomes on the high-grade histological subtypes of ovarian cell lines namely OVCAR5, SKOV3 and Kuramochi showed effective in inhibiting cell viability in all three cell lines. Overall, this work establishes a reliable microfluidic platform for producing homogeneous liposomal systems with potential applications in targeted drug delivery.

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Disclaimer

This manuscript has undergone grammar and syntax correction using ChatGPT, an AI language model developed by OpenAI. While every effort has been made to improve the clarity and accuracy of the language in this document, the authors acknowledge that the final content and scientific interpretations remain the sole responsibility of the authors and their collaborators. ChatGPT has been used exclusively to enhance the manuscript's readability and expression, and not to generate any type of data.

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Prospects: Advantages and Drawbacks of the delivery systems

Among the three delivery platforms explored in the thesis, each embodies a distinct balance of technological elegance, green-chemistry credentials, and translational practicality determines its promise for nutraceutical or pharmaceutical use. Supercritical-CO₂ (Sc-CO₂) derived quercetin nanocrystals stand out for their sustainability profile: CO₂ is inexpensive, non-flammable, easily recycled, and leaves no residual organic solvent—an advantage of special regulatory significance for food or supplement applications. The rapid antisolvent precipitation, coupled with Pluronic F127 and 2-HP- β -cyclodextrin surface chemistry, yields particles in the range of 200-300 nm with clean FTIR/XRD fingerprints, a three-order-of-magnitude jump in apparent solubility, and heat stability up to 100 °C. Yet the same supercritical framework imposes a steep learning curve and capital cost, demands compressible-fluid-compatible pumps and pressure vessels, and can suffer particle aggregation once depressurization ceases if the steric stabilizer layer is sub-optimal. Moreover, while the nanocrystal approach boosts dissolution, it relies on passive diffusion across the gut wall but without a carrier matrix, there is no intrinsic protection against intestinal efflux transporters or first-pass metabolism. In contrast, quercetin liposomes fabricated from soy-derived lecithin enriched with β -sitosterol are wholly food-grade, GRAS components, reinforcing their identity as a nutraceutical rather than a drug. Soy phosphatidylcholine lends fluid, unsaturated bilayers that remain liquid-crystalline at room temperature, improving palatability and enabling cold-chain-free distribution contributes an economic plus for large-volume supplement markets. β -Sitosterol, a phytosterol structurally akin to cholesterol, stiffens the membrane just enough to curb leakage while adding its own LDL-cholesterol-lowering health claim, a neat case of “excipient value adds.” The trade-off is shelf-life: poly-unsaturated fatty acyl chains in soy PC oxidize readily, and sitosterol offers less oxidative protection than true cholesterol, so lyophilization, spray drying, or nitrogen-flushing are mandatory. In vivo, the absence of stealth agents such as PEG means these vesicles are rapidly cleared once systemic, but that is acceptable indeed desirable for an orally delivered antioxidant supplement where systemic circulation is not the primary goal.

VS10 liposomes built from hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PEG-2000-DSPE embody a clinic-ready “stealth” architecture optimized for intravenous administration as a chemotherapeutic. HSPC has saturated C16/C18 chains that pack tightly, raising the gel-to-liquid crystalline transition ($T_m \approx 55$ °C) and conferring formidable physical and chemical stability. Cholesterol intercalates to fill voids, damping the permeability that otherwise accompanies gel-phase lipids, while PEG-DSPE projects a hydrophilic corona that

minimizes opsonization and extends half-life which is critical for exploiting the enhanced permeability and retention (EPR) effect in solid tumors. These benefits come with cost (pharmaceutical-grade HSPC and PEGylated lipids are expensive), manufacturing complexity (remote-loading or ethanol-injection is usually combined with sterile filtration and lyophilization), and a residual risk of complement-activation-related pseudo-allergy (CARPA) inherent to PEGylated carriers. Yet for a BCS class 2 anticancer drug like VS10, whose therapeutic index hinges on selective tumor exposure, the pharmacokinetic gain decisively outweighs such liabilities.

In summary, Sc-CO₂ nanocrystals emerge as the front-runner when “green label” extraction and simple oral-solid-dose conversion are paramount meanwhile the Quercetin-soy-lecithin- β -sitosterol formulation excels where regulatory simplicity, consumer perception, and cost-efficiency dominate, but its unsaturated matrix and non-stealth nature limit systemic ambitions. The HSPC/cholesterol/PEG-DSPE construct, by contrast, delivers pharmaceutical-grade stability, prolonged circulation, and tumor targeting, albeit at higher economic and regulatory cost.

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