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Review

Advanced nanotechnologies for enhancing the bioavailability of silymarin: a state of the art

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Abstract: Silymarin, a mixture of flavonolignan and flavonoid polyphenolic compounds extractable from the milk thistle seed, Silybum marianum, has anti-oxidant, anti-inflammatory, anti-cancer and anti-viral activities potentially useful in the treatment of several liver disorders, such as chronic liver diseases, cirrhosis and hepatocellular carcinoma. Equally promising are the effects of silymarin in protecting the brain from the inflammatory and oxidative stress effects by which metabolic syndrome contributes to neurodegenerative diseases. However, despite clinical trials have proved that silymarin is safe at high doses (>1500 mg/day) in humans, it suffers limiting factors such as low solubility in water (<50 μg/mL), low bioavailability and poor intestinal absorption. To improve its bioavailability and provide a prolonged silymarin release at the site of absorption, the use of nanotechnological strategies appears to be a promising method to potentiate the therapeutic action and promote sustained release of the active herbal extract. The purpose of this study is to review the different nanostructured systems available in literature as delivery strategies to improve the absorption and bioavailability of silymarin.

Keywords: Silymarin; silybin; nanoemulsion; solid lipid nanoparticles; nanostructured lipid carriers; liposome; polymeric particles; self-emulsifying delivery systems; enhanced bioavailability

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Contents

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1. Introduction

Nanotechnology has become an important part of medical science, pharmaceutical and food industry, since it focuses on the development of novel delivery systems to sustain or prolong drug release properties, control human biological systems at the molecular level and improve the therapeutic activity of various types of substances and compounds [1]. The usefulness of nanotechnology has also been extended to natural healthy products, where numerous efforts are continuously being made to improve the bioavailability and therapeutic potential of active compounds extracted from natural sources [2]. Nanotechnology is defined as a process by which some main physicochemical properties of particles are changed by reducing their size to nanodimension. The various definitions of nanoparticle size range are presented in different industries such as food, pharmaceutical, and cosmetics. For instance, in the food industry, European Food Safety Authority (EFSA) has called the particles below 100 nm as nanoparticles. However, in general, particles with sizes less than 1000 nm can be considered as nanoparticles [3]. Nanoencapsulation, a main branch of nanotechnology, can be applied by two common methods including "bottom-up" (self-assembly and self-organization) or "top-down" (physical processing). Nanoencapsulation of natural compounds provides a large surface area and causes steerable release, protects them against different stresses during the processes and storage in comparison to their microencapsulation, and improves their bioavailability for removing free radicals, antidisease and antimicrobial activities. Indeed, the bioavailability of plant extract can be increased with higher solubility, absorption, and permeation of them in the body and food formulations through nanoencapsulation process. For instance, nanoencapsulation allows phenolics and antioxidants to be absorbed passively from the lumen of the intestine into the lymphatic and blood circulatory system; therefore, their bioavailability is increased [4]. It should be also mentioned that selection of a nanoencapsulation technology depends on several parameters, such as physicochemical features, required particle size, release type, delivery method, process cost, etc. Hence, a vast arsenal of increasingly sophisticated nano-methodologies is now available for researchers to efficiently entrap and make more bio-available *in vivo* poorly water-soluble active plant ingredients, among which a very well documented example concerns silymarin. Indeed, inadequate aqueous solubility of active pharmaceutical ingredients (APIs) is a major concern in the formulation and development of novel delivery systems since it directly affects the bioavailability [5]. Thus, novel formulations of silymarin have been prepared by innovative techniques and found to increase its therapeutic efficacy against various diseases [6]. Several reviews have appeared on the subject in the recent past, each of them trying to offer the reader a broad and increasingly updated vision of the most advanced nanoencapsulation solutions developed to increase the *in vivo* absorption efficiency of silymarin [7-11]. The ambition in the present paper has been to summarize and focus on the advances made over the past 15 years regarding the nanoencapsulation of silymarin, as well as on the emerging trends of the nano-delivery of this drug in new pharmaceutical applications.

2. Silymarin: source and physicochemical properties

Silymarin consists of several flavonoid-like compounds extracted from the small hard fruits (kenguil seeds) of *Silybum marianum* L. Gaertn (milk thistle), which grows extensively in Europe and Asia including India. The drug belongs to a class of compounds − flavonolignans − likely produced in the plant by radical coupling of flavonoid and coniferyl alcohol [12]. Silymarin is a complex mixture of four flavonolignan isomers: silybin (70-80%), silychristin (20%), silydianin (10%) and isosilybin (0.5%), which are assumed to be responsible for the therapeutic liver-protecting activity of the extract [13-16]. For the main component silybin, a large amount of pharmaco-toxicological and clinical documentation exists [17-19]. The molecular structure representative of one of the two diastereoisomers (silybin A and silybin B, 1: 1 mixture) is shown in Figure 1.

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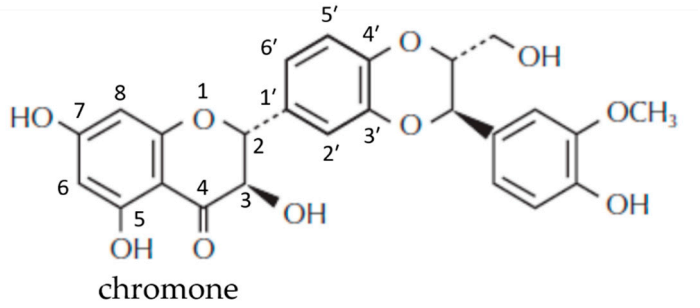

Figure 1. molecular structure of Silybin A

Its molecular structure possesses a chromone fragment responsible for weak acidic properties, enabling donor-acceptor interactions with basis. The presence of polyphenol hydroxyls and the ability to form complexes with transition and other metal ions in the 3,4- or 4,5-positions, confers high antioxidant capability to the molecule. Several studies have shown that for this active ingredient even very high doses are well tolerated by animals and humans [20,21]. In particular, the oral 50% lethal dose is 10,000 mg/kg in rats while the maximum tolerated dose is 300 mg/kg in dogs [22]. Due to its lipophilic nature characterized by a log*P* value of 1.41 [23] where *P* is the partitioning coefficient of the drug, the therapeutic efficiency of silybin is rather limited by its very low water solubility (430mg/L) [24-27]. According to the biopharmaceutical classification system (BCS), silymarin belongs to class II BCS, which includes either insoluble compounds in aqueous media or which have a very low solubility. Consequently, the drug is poorly absorbed (20-50%) from the GIT and has a low bioavailability from oral formulations [28]. Several semisynthetic compounds have been designed to overcome the drawback of very low water solubility such as, e.g., the bis-hemisuccinate (Legalon®), 23-O-phosphate, 23-O-β-glycosides derivatives and silybinic acid [29]. However, chemical modifications leading to an increase in silybin water-solubility usually led to an impairment of its antioxidant (antiradical) activity [24]. The hepatoprotective mechanism of silymarin and its main component silybin is due not only to the antioxidant activity but also to a membrane-stabilizing action that prevents or inhibits the lipid peroxidation process [30].

3. Types of Delivery Systems (DSs) designed to encapsulate Silymarin/Silybin

In the following subsections, various classes of DDS specifically designed for silymarin encapsulation will be reviewed and systematically classified in the final Table 1, including the suitable cross references mentioned in the text. Henceforth, the acronym SIL will be used indifferently to indicate both silymarin and its main and biologically active constituent, silybin. The meaning of the other acronyms is shown in the list of abbreviations at the end of the review.

3.1. Lipid-based formulations

A widespread formulation strategy exploits lipid-based colloidal vehicles as winning option for the delivery of SIL penalized by the poor aqueous solubility. Lipid-based formulations (incorporation of the active lipophilic component into inert lipid vehicles) are used to improve the oral bioavailability of poorly water-soluble drug compounds, which include micro or nanoemulsions, oils, self-emulsifying formulations, surfactant dispersions, proliposomes and liposomes, solid lipid nanoparticles and lipid nano carriers, etc. These lipidic formulations can be broadly divided into two groups namely liquid Oil-in-Water (O/W) emulsions (LEs) and solid lipid nanoparticles (SLNs). The LE systems could be lipid solutions, emulsions, microemulsions and self-(nano/micro) emulsifying drug delivery systems. SLNs are novel lipid-based formulations which are constituted exclusively of

biodegradable lipids such as highly purified triglycerides, monoglycerides, hard fats, complex glyceride mixtures or even waxes, which are solid at physiological temperature.

3.1.1. Liquid Emulsions (LEs)

The formulations based on both microemulsion and nanoemulstion strategies for oral administration, are designed with food-acceptable components or generally recognized as safe (GRAS) to increase solubility, stability, and improve the SIL permeability. Micellar carriers have been found useful to encapsulate efficiently SIL and enhance its water solubility and bioavailability. For example, due to the physiological compatibility and solubilizing capacity, the PC-BS mixed micelle was found to be a good nanocarrier candidate to encapsulate SIL with high loading capacity. Yu et al. [31] prepared phospholipidic micelles mixed with BS (SPMM) to solubilize SIL in their inner hydrophobic cores. From pseudo-ternary phase diagram investigations, an optimum micellar formulation was capable to solubilize the drug up to 10 mg/ml, which was also found to double in the presence of PVP [32]. An analogous nano-vehicle for the water-insoluble SIL purposely designed for parenteral application was proposed by Duan et al. [33], who first prepared a SIL-PPC equimolar complex by TFD method, which was then dissolved in anhydrous ethanol together with PPC and SDC at various molar ratios. After evaporation of the organic phase, the mixed micelles loaded with SIL were reconstituted by adding double distilled water. The optimal formulation led to a SIL loading efficiency of 14.43%, which corresponded to a drug solubility in water of 10.14±0.36 mg/mL.

A similar strategy was pursued by Xu et al. [34] who first prepared a SIL-PC complex at a mass ratio of 1:3 by TFD method, which was subsequently dissolved in anhydrous ethanol together with a water-soluble derivative of vitamin E (TPGS) and PC at ratios 4:1:20, w/w/w. After ultrasonication and further solvent evaporation, the resultant lipid film was hydrated in distilled water leading to a lipid suspension, which was sonicated until a translucent colloidal dispersion was obtained. The aim was to test the anti-metastatic effect of SIL in combination with TPGS co-entrapped in lipid nanoparticles, to suppress effectively the metastasis of breast cancer both *in vitro* and *in vivo*. The average particle size was about 45 nm with a zeta potential value of 2.78 ± 0.31 mV. The encapsulation efficiency of SIL in lipid nanoparticles was approximately 99%. The *in vivo* results supported the potential use of SIL-TPGS loaded NPs as an anti-metastasis agent capable to inhibit the invasive and metastatic activities of breast cancer cells instead of acting a cytotoxic effect against them.

Other types of O/W microemulsions were formulated to incorporate 2% w/w of drug as a potential dermal delivery system. In particular, microemulsions containing IPM as oil phase emulsified with a 1:1 mixture of water-dispersible Labrasol® and HCO-40® nonionic surfactants and Transcutol® as cosurfactant, enhanced SIL solubility while maintaining adequate physical and chemical stability [35].

SIL-loaded lipid O/W emulsions were optimized by testing the emulsification properties of soybean lecithin as surfactant and Tween 80 as cosurfactant in combination with several food grade oils (soybean oil, castor oil, and olive oil) [36]. The authors reported that SIL was added to the lipid phase in soybean oil as 10% aqueous solution in 1 M NaOH up to an optimum drug loading of 1 % w/w to produce an emulsion stable for 35 days, constituted by oil droplets with size distribution range of 0.31-1.24 μm and median diameter of 0.46 μm. The *in vitro* drug release behaviour was faster from the SIL-loaded lipid emulsion as compared with a SIL propylene glycol solution.

To overcome the disadvantage of the administration of large volumes of (micro)emulsions per dose, several researchers focussed their studies on the design of preconcentrated organic liquid phases loaded with poorly water soluble drugs, capable of reconstituting the (micro)emulsion spontaneously once in contact with an aqueous milieu (gastric fluids after ingestion). The resulting formulation is commonly termed in literature as liquid Self-Emulsifying Drug Delivery System (SEDDS) [37,38]. The essential property that must be satisfied in the development of a liquid SEDDS formulation is that the drug must remain partitioned within the O/W droplets after dilution with the aqueous medium in the GIT. Otherwise, the drug could undergo an unwanted precipitation that would lead to poor bioavailability in vivo. Among the first documented studies on the application of SEDDS as potential nanocarrier to increase SIL solubilization and its oral bioavailability, we mention

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the work by Wu et al. [39], who optimized the multi-component oily phase, ethyl linoleate/Tween80/ethyl alcohol, to solubilize SIL. The self-emulsifying properties of that system were tested upon titration with various aqueous media until a stable O/W microemulsion was obtained. During the titration, the samples were agitated gently in order to reach equilibrium quickly. Dilution volume had no significant effect on droplet sizes (mean value of dozens nm), which were found also unaffected by the increase of drug loading up to 100 mg of SIL per 1 g of oil phase. Relative SIL bioavailability after oral administration of the said lipid emulsion, improved approximately 1.88- and 48.82-fold that of drug dissolved in PEG 400 solution and aqueous suspension, respectively.

Another optimized SEDDS was prepared using 10 % GMO as the oil phase and 15% SIL, with 37.5% of a surfactant mixture of TWEEN 20 and HCO-50 (1:1), and 37.5% Transcutol® as cosurfactant [40]. The authors evaluated also the SIL solubility in various solvents at 25°C. The O/W microemulsion was generated upon water addition until reaching a maximum water content of 95.4 %. After aqueous dilution, the mean droplet size of the internal oil phase was about 67 nm. The release rate of the drug from the SEDDS measured through *in vitro* dissolution tests, was approximately 2.5 times higher than that from the reference commercial product (Legalon®). After oral administration, SIL-loaded SEDDS showed a 360% higher bioavailability compared with the reference formulation.

In a similar subsequent investigation, Li et al. optimized SIL-loaded SEDDS formulations based on ethyl linoleate, Cremophor EL and ethyl alcohol, which were selected regarding the self-microemulsifying ability, solubilization ability, and reduced use of surfactant, [41]. The best combination of ingredients screened after a systematic pseudo-ternary phase diagram study, yielded a SIL solubility of 130.8 mg/mL homogeneously dispersed in small O/W droplets with mean size in the range 20 - 30 nm and no changes were detected at 40°C for 3 months. Both the *in vitro* release performance and *in vivo* bioavailability after oral administration of SIL from SEDDS were evaluated and compared with the commercial SIL preparation Legalon®. The relative drug bioavailability of SEDDS to commercial SIL suspension was 227%.

To overcome the side effects caused by high surfactant levels usually employed in SEDDS, Wei et al., designed a supersaturable SEDDS (S-SEDDS) formulations to improve SIL oral bioavailability [42]. It consisted of a reduced amount of surfactant in combination with HPMC added in the liquid SEDDS to induce a supersaturated state *in vivo* by preventing or minimizing the SIL precipitation. The authors evaluated also the SIL solubility in various oils, surfactants and cosurfactants at 25°C. Labrafac® CC showed the highest drug solubility and was selected as an oil phase for the formation of S-SEDDS. Cremophor RH 40 was chosen as a surfactant for its good emulsion-forming ability and smaller droplet size of the optimized SIL loaded emulsion (~ 50 nm), thanks also to the synergic effect of the compresence of Transcutol® and Labrasol® as cosurfactants. From *in vitro* studies, it was confirmed the stabilizing effect of HPMC in maintaining high SIL solution concentrations (supersaturated state). Precipitates collected after the *in vitro* tests from the S-SEDDS formulated with HPMC, were identified as amorphous SIL while crystalline precipitates were found when HPMC was absent in the formulation. Relative drug bioavailability after oral administration of a SIL dose of 533 mg/kg was found higher for S-SEDDS than SEDDS, i.e., same formulation without HPMC. In particular, C_{max} was 16.1 μg/mL as compared to that of the SEDDS formulation (5.68 μg /mL), while AUC of SIL from S-SEDDS was approximately 3.0-fold higher than that of SEDDS.

Stable nanoemulsions for SIL delivering and containing Labrafac® as an oily phase, Solutol® HS 15 as surfactant, Transcutol® as co-surfactant, and water as aqueous phase were developed by Adhikari et al. [43], to test the radioprotective potential of the SIL-loaded nanosuspensions against γ-radiation-induced oxidative damage in human embryonic kidney cells. The formulations were designed to act as SEDDS after water addition, until the oil phase was 10 – 15 % at the end of the dilution process. HEK cells viability upon treatment with variable concentrations of SIL-SEDDS and bare SIL suspensions as control was checked prior to irradiation. Radiation-induced apoptosis was estimated by microscopic analysis and cell-cycle estimation. The proposed formulation based on SEDDS technology to improve the SIL bioavailability was found radioprotective, supporting the possibility of developing new approaches to radiation protection via colloidal dispersions of SIL.

A very stable O/W nanoemulsion consisting of silymarin solubilized in nanosized oil droplets dispersed in an aqueous medium by a mixture of Tween 80 and ethanol as co-surfactant, was optimized through a systematic investigation on experimental pseudo-ternary phase diagrams [23]. Among various tested oils such as OA, IPM, Triacetin, the highest value of SIL solubilisation was observed in Sefsol-218 (183.375 ± 0.0036 mg/mL), which was selected for the nanoemulsion formulation at 5% w/w in water. After oral administration of SIL solubilized in the nanoemulsion, AUC and C_{max} were, respectively, 199.45±56.07 μg h/mL and 31.17±7.56 μg/mL, namely, 4-fold and 6-fold higher than those of the correspondent drug aqueous suspension.

A SIL-loaded liquid nanoemulsion was formulated by Yang et al. [44], using the SPG membrane emulsification technique and then spray-dried to obtain solid state nanoparticles. Dissolution, bioavailability, and hepatoprotective activity *in vivo* were assessed by comparison with a commercially available SIL-loaded product. Optimal formulation was composed by SIL, castor oil, PVP, Transcutol®, Tween 80, and water at the weight ratio of, respectively, 5:3:3:1.25:1.25:100. The mean sizes of the SIL-loaded nanoemulsion and nanoparticles obtained after spray-drying were about 170 and 214 nm, respectively. The SIL bioavailability after oral administration from the nanoparticles was about 1.3-fold higher than that obtained with a commercial product.

In a more recent investigation, SIL enriched nanoemulsions were formulated using different oils such as sunflower, EVO and castor oils, respectively, [45]. SIL solubility in castor oil was 0.668 ± 0.072 mg/g whereas in EVO and sunflower oils was rather lower. However, upon addition of Tween 80 in oil (10 mg/g), drug solubility increased up to reach 1-2 mg/g in the three tested oils. Coarse emulsions were first prepared by mixing 200 g oil+SIL with 1 L of aqueous phase and then subjected to HPH, giving rise to final droplet sizes in the range 200-300 nm. Moreover, it was found that the greater the oil susceptibility to oxidation, and thus the formation of oxidation products, the greater the SIL degradation incorporated into nanoemulsions.

In another recent study, Nagi et al. employed a Box-Behnken statistical design (BBD) to optimize SIL-loaded nanoemulsions using Capryol 90 as oil phase capable of solubilizing SIL up to 40.00 ± 1.53 mg/mL, in terms of various factors such as processing pressure and number of cycles of HPH technique and amount of surfactant/cosurfactant mixture [46]. The non-ionic hydrophilic surfactant Solutol® HS 15 was selected due to its high capacity to solubilize hydrophobic drugs and low toxicity (LD50 > 20 mg/kg) [47], and used in combination with Transcutol® selected as cosurfactant on the basis of the good miscibility with Capryol 90 and Solutol® HS 15. The optimal nanoemulsion satisfying the criteria of low droplet size and low PDI, necessary for high drug release potential and drug permeation through the GIT membrane, was characterized by nanodroplets of about 50 nm (PDI: 0.45) and zeta potential -31.49 mV. The values of AUC and C_{max} of that nanoemulsion formulation after oral administration were found equal to 28.69±3.28 μg h/mL and 3.25±0.48 μg/mL, respectively, i.e., 1.9-fold and 2.7-fold higher than those of a marketed drug suspension.

In a recent study [48], it was reported that a SIL commercial extract could be completely solubilized at the dosage of 40 mg/mL in a nanoemulsion formulated using 2.5 g of Labrasol® (20%) as the oil phase and 2.5 g of Cremophor® EL/Labrafil® as the surfactant/cosurfactant mixture in a 1.5:1 ratio, the remaining mass being deionized water (60%). Besides, the authors determined selectively the solubility of the main constituents identified in SIL extract, such as, TXF, SILcr, SIL and isoSIL, in various oils, surfactants, and cosurfactants to ascertain the appropriate components of the nanoemulsions. The SIL extract loaded within O/W nanodroplets showed excellent physical and chemical stability, as the size (30-40 nm) and PDI (0.114-0.179) were unaffected and no degradation of active constituents was recorded over 40 days of observation. *In vitro* permeation studies were performed to determine the suitability of the prepared nanoemulsion for oral delivery.

3.1.2. Liposomes

Liposomes are hollow spherical nanoparticles with a closed shell of a lipid membrane (mono- or multi-layer), inside of which an aqueous solution can be encapsulated. These supramolecular aggregates owe their success as carriers of therapeutic drugs for many advantages including the capability to encapsulate both hydrophilic and lipophilic drugs, having targeting and controlled

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release properties, cell affinity, tissue compatibility, reduced drug toxicity and improved drug stability [49]. Moreover, liposomal systems are known to find an immediate access to the reticulo-endothelial system (RES) rich sites like liver and spleen, and this self-targeted nature of liposomal carriers can be exploited well for drug distribution to hepatic site. During the researches, the conventional structures of the liposomes have been subjected to several changes, which have brought out a series of new type liposomes, such as long-circulating stealth liposomes, stimuli-responsive liposomes, cationic liposomes and ligand-targeted liposomes. Liposomes can be prepared using a wide range of methods, such as thin-film dispersion (TFD), reversed-phase evaporation (RPE), alcohol injection, and spray-freeze-drying. Other strategies comprise proliposomal formulation, with the use of a cryoprotectant and high process temperature, and supercritical fluid of carbon dioxide method (SCF-CO_2), which is a flexible and environmental-friendly technique by which particle sizes and shapes can be controlled by tuning the experimental conditions (temperature and pressure).

One of the first liposomal formulation of SIL reported in literature dates back to the early 2000s. The study addressed by Maheshwari et al. [50], focused on the development of factors such as the drug to lipid ratio, the proportion of CHOL and presence of the charge inducer DCP in the optimization of the formulation loaded with SIL. The highest drug entrapment of 94.7 % was achieved in the formulation with SIL / PC / CHOL / DCP ratio of 2 : 10 : 2 : 1. The obtained size range of SIL loaded liposomes (56−1270 nm with median diameter of 390 nm) was suitable for i.v. administration for hepatoprotective studies in mice. A drug leakage of about 40 % was observed in 28 days as well as evident aggregation phenomena recorded after 3 weeks of the preparation.

To overcome instability problems that commonly occur in the GIT and improve the poor aqueous solubility of SIL, El-Samaligy et al. [51], investigated the feasibility of encapsulating the drug in a liposomal dosage-form for buccal administration via spray. Liposomes were prepared by RPE method using a base lipid mixture of soybean lecithin and CHOL in a 9:1 optimized molar ratio. In addition to the basic liposome ingredients various additives were gradually introduced, such as positively (SA) or negatively (DCP) charge inducers and non-ionic surfactants (Tween 20 or Tween 80). At the end of a multifactorial screening, an optimal composition for hybrid liposomes was derived as lecithin / CHOL / SA / Tween 20 at 9:1:1:0.5 molar ratios, which warranted both best SIL encapsulation efficiency of about 69% and high *in vitro* absorption and permeation performances [52].

In order to improve the stability of liposomal nanocarriers and enhance the SIL encapsulation, Xiao et al. [53] adopted the strategy of "proliposomes", which are defined as dry, solid particles that form a reconstituted liposomal suspension when put in contact with water [54]. The SIL-proliposomes were prepared by film-deposition using mannitol as carrier and a mixture of methanol and chloroform (2:1 v/v) as the apolar medium to dissolve SIL and phospholipids. The content of SIL in the proliposomes was 9.73 % (w/w). In the reconstituted liposomal suspension, the mean particle size was 196.4±43.7 nm while a mean value for SIL entrapment efficiency was 92.56±0.93%. After oral administration in beagle dogs of SIL entrapped in the reconstituted liposome suspensions (drug equivalent to 7.7 mg kg^{-1}), the pharmacokinetic parameters AUC and C_{max} were, respectively, 2.46±0.58 μg h/mL and 0.47±0.13 μg/mL.

The proliposome nanotechnology was also exploited to improve the water solubility and bioavailability of 2,3-dehydrosilymarin, an oxidized form of SIL characterized by significantly greater antioxidant and anti-cancer activity than the reduced precursor [55]. Hence, the 2,3-dehydrosilymarin-loaded proliposome powder was produced by the TFD-freeze drying method obtaining a polyphase dispersed system composed of phospholipids, CHOL, IPM and sodium cholate, with optimal drug-lipid ratio set to 1:3 [56]. The correspondent drug content after reconstitution with water was 25.00±5.93 μg/mL, yielding an encapsulation efficiency of 81.59%±0.24, which was predominately dependent on the drug/phospholipid and sodium cholate/phospholipid ratios. The improved oral absorption in rabbits was ascribed to the relatively small size of liposomes distributed in the range 7 - 50 nm and average diameter of about 16 nm. The AUC and C_{max} were approximately 2.29-fold (12.77±1.39 μg h/mL) and 4.96-fold (2.83 μg/mL) higher than those of the simple 2,3-dehydrosilymarin suspension.

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SIL-loaded PEGylated liposomes decorated with the hepatic targeting ligand Sito-G (a carbohydrate epitope) were prepared by TFD method, [57]. The liposomes contained fixed 2:1 molar ratios of HSPC and CHOL with or without PEG as well as a varying concentration of Sito-G to check its optimal amount for improving the targeted delivery of the encapsulated SIL to hepatic target cells. The combination of relatively high melting point HSPC with a high percentage of CHOL provided liposomes with very rigid lipid bilayers. The obtained multilamellar vesicles were exposed to several freeze-thaw cycles in which the liposomes were freezed in liquid nitrogen for 2 min and defrost in warm water bath for 2 min. The submicron-sized liposomes were prepared by using a pressurized extruder with two polycarbonate membrane filters with pore size of 100 nm. The final lipid concentration in the liposome formulations was 4 mg/ml. The particle size distribution of the liposomes with mean values in the range 145-168 nm showed very good homogeneity (PDI 0.15-0.3). An acceptable drug encapsulation efficiency was recorded for all tested formulation (about 60 % in average), although high contents of Sito-G were found detrimental to the stability of liposomal membrane, thus leading to a decrease in the encapsulation efficiency. A systematic investigation of the in vitro release profiles performed with the dialysis method (37 °C in HEPES buffer, pH = 7.4), indicated that PEGylated liposomes exhibited a sustained release of SIL as compared to non-PEGylated liposomes. On the other hands, PEGylation of liposomes equipped with Sito-G manifested a reduced SIL cellular uptake with HepG2 cells compared to non-PEGylated nanocarriers.

A more recent development of using PEGylated liposomes to improve the SIL bioavailability is reported in the work published by Ochi et al. [58], who demonstrated a synergic effect on the liver cancer cell line HepG2, provided by the co-encapsulation into PEGylated nano-liposomes of SIL and GA. The liposomial suspensions, prepared by TFD method followed by sonication, were formulated with SIL and GA at a 1.74:1 molar ratio together with a mixture of DPPC, CHOL, and mPEG2000-DSPE at a specified molar ratio. Scanning Electron Microscopy analyses showed that the co-encapsulated nano-liposomes had a mean diameter of 43 nm while zeta potential was -23.25 mV, sufficient to inhibit liposome aggregation.

Beside to enhance the SIL bioavailability, the liposomal formulation developed by Kumar et al. [59], was designed to promote the regeneration of hepatocytes and to prevent inflammation in liver. The drug entrapment efficiency of liposomes prepared with the TFD technique, was found to be maximum (55 %) for formulation containing SPC and CHOL at molar ratio 6:1. The optimal liposomal formulation yielded a 3.5-fold higher bioavailability of SIL (AUC 0.500±0.023 μg h/mL) than the correspondent drug suspension. Likewise, C_{max} was found 5.25-fold (0.716±0.043 μg/mL) higher than the SIL suspension. Analysis of *in vivo* studies suggested that SIL encapsulated in said liposomal carriers might have targeted inflammatory cells resulted in increased anti-inflammatory activity.

A slightly different approach was explored by Angelico et al. [60] in the preparation of liposomes loaded with SIL-phytosome rather than using the commercial purified SIL extract in the formulation. The phytosome unit is a molecular complex between phospholipids and standardized polyphenolic constituents in a 1:1 or 2:1 molar ratios [61], and according to numerous studies, it proved to be more bioavailable compared to the purified molecular extracts though most of formulations have been addressing to orally and topically drug administrations [62,63]. The addition of lecithin in the starting lipid film based on SIL-phytosome such that the final phospholipid/SIL ratio reached 6:1, yielded stable phyto-liposomes with the suitable surface charge and average dimensions in view of a potential parenteral i.v. use, where the nanoparticle size is a critical parameter to be controlled. The cellular uptake of SIL encapsulated into phyto-liposomes and its antiviral activity were also tested *in vitro* with Huh7.5 cells [64]. The data clearly demonstrated that the cell absorption was 2.4-fold more efficiently than free SIL, and 300-fold more potent pharmacological activity. It is worth noting that the phyto-liposomes were able to reduce the hepatitis C virus infection by inhibiting the entry of viral particles into cells.

Methods that use SCF-CO_2 technique have been also employed for the preparation of SIL-loaded liposomes. In particular, the solution-enhanced dispersion by supercritical fluids (SEDS) has been adopted to produce liposomes constituted by HSPC and SGC as base lipid ingredients, [65]. The optimized product provided better performances than liposomes prepared with more conventional

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methods such as RPE or TFD. Based on the analysis of drug release profiles *in vitro*, the novel formulation improved the SIL solubility. *In vivo* tests showed an improved oral bioavailability of drug administered in liposomes containing BS (AUC 18.406±1.481 μg h/mL; C_{max} 1.296±0.137 μg/mL) compared to an aqueous suspension or a commercial SIL product.

A recent study reported on the effect exerted by SIL contents and type of BS on the drug encapsulation efficiency in SIL-loaded BS-liposomes (bilosomes) prepared with TFD method [66]. The obtained vesicle dispersions were characterized by highly negatively charged zeta-potential values compared to an analogous preparation formulated with CHOL in place of BS. Considering particle dimensions, BS/liposomes containing SC exhibited the largest particle diameter (595.1 ± 98.48 nm) among all BS investigated. Among all the screened formulations, the optimum composition in terms of highest SIL-entrapment efficiency (84.54 %) corresponded to a lipid molar ratio 4:1 for SPC / SC system. *In vitro* release studies revealed biphasic pattern of all formulations while *in vivo* investigations revealed that bilosomes showed a pronounced effect in retaining the hepatoprotective as well as oxidative stress biomarkers to their normal levels against CCl_4 induced hepatotoxicity.

3.1.3 Solid-Lipid Nanoparticles (SLNs), Nanostructured Lipid Carriers (NLCs)

Solid lipid nanoparticles (SLNs) are the first generation of lipid-based nanocarriers that are formulated from lipids, which are solid in the body temperature and stabilized by emulsifiers [67]. Emulsomes are special case of SLNs, considered as the solid state version of common uninamellar and multilamellar lipid vesicles, i.e., nanoparticles with an internal solid fat core surrounded by one or more phospholipid layers [68]. For an exhaustive discussion about pros and cons occurring in the use of this type of lipid nanocarriers see, e.g., a recent review by Ghasemiyeh et al. [69]. Differently from matrices having either solid (SLNs) or liquid lipids (LEs) as core composition, the nanostructured lipid carriers (NLCs), which belong to the second generation of lipid nanoparticles, represent hybrid formulations prepared by blending solid lipids and liquid lipids, thus resulting in a less ordered inner structure [70].

Considering the application of these drug delivery nanotechnologies as nano-vehicles for SIL, in an interesting work by Shangguan and coworkers [71], it was reported the performance in animal models of both SIL-loaded SLNs and SIL-loaded NLCs, by comparing their oral bioavailability with that of their lipolysate counterparts and fast-release formulations. The goal was to determine whether and to what extent the integral lipid nanoparticles contribute to the overall bioavailability of SIL selected as a poorly water-soluble model drug.

In an earlier study, SIL-loaded SLNs were developed using the emulsifiers Compritol 888 ATO, soybean lecithin and poloxamer 188 and both hot and cold variants of the HPH method were compared each other to check differences in drug incorporation modes and release mechanisms [72]. From the analysis of results obtained by centrifugal ultrafiltration method, the SIL encapsulation efficiency in the cold preparation reached 87% with a fraction of adsorbed drug of about 8%, while for hot homogenization the entrapment efficiency was 43% and the fraction adsorbed was 54%. The *in vitro* tests carried out by reverse dialysis bag technique at pH 7.4, showed a prolonged drug release for SIL-SLNs produced by cold homogenization. *In vivo* studies confirmed the trend showing higher drug levels and longer residual time in the plasma and liver after oral administration of the cold preparation compared to SIL aqueous suspension.

Experimental evidences of the anti-hepatotoxic property of SIL encapsulated in SLNs were reported in Cengiz et al. [73], who tested their formulations against the liver damage induced in model animals by the administration of a combination of the hepatotoxin D-GaIN and TNF-α. The solid lipid nanosuspensions based on Compritol and Tween 80, respectively, lipid matrix and surfactant, were prepared through the hot homogenization technique. The resultant colloidal SIL-loaded dispersions were characterized by particle sizes varying in the range 165 - 200 nm with zeta potential of – 26.5 mV. Both in vitro and in vivo tests demonstrated that SIL-loaded SLNs were found to be more effective than bare drug as control in curing liver damage, mainly owing to the slow and regular release of SIL by NPs, which in turn could have increased the drug bioavailability and then its therapeutic effects.

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To reduce the disadvantages due to the elimination from blood circulation of SLNs by the reticuloendothelial system (RES), Zhang et al. [74] proposed a novel formulation based on stearic acid covalently grafted with PEG 1000 (Brij 78) as stealth agent to form a hydrophilic steric barrier around the SLNs. SIL was incorporated into stealth SLNs according to the EES method, which consisted in the preliminary preparation of a lipid emulsion, obtained by adding dropwise an organic phase of steric acid and SIL dissolved in acetone to an aqueous phase containing the non-ionic surfactant Brij 78. After removing the organic solvent by evaporation, the resulting emulsion system was quickly added to a given volume of cold distilled water to obtain a final suspension of SIL-loaded SLNs. The authors observed that the rate of addition of organic phase to the aqueous phase was the crucial step in preparing the emulsion. A systematic investigation was carried out in order to optimize the drug entrapment efficiency and the homogeneity of particle size distributions. The mean particle size of the optimized formulation was 179 nm (PDI: 0.168) and zeta potential of -25 mV, which represented quality values of good stability. *In vitro* assays by dialysis method revealed a very slow drug release, a property considered beneficial for SIL bioavailability after oral administration.

Thanks to the good biocompatibility and biodegradability, the emulsomes nano-encapsulation technology has been recently applied to provide particle stability, high entrapment efficiency, and sustained SIL release, [75]. The preparation followed the TFD method with the variant of choosing a solid lipid matrix at r.t. such as triglycerides composed of natural unbranched fatty acids or glycerol triesters of saturated fatty acids (e.g. trilaurin), in place of biocompatible oils as internal phase in which SIL has to be dissolved. Other ingredients were CHOL and Tween 80 mixed together with trilaurin and PC in chloroform solution. The film obtained after solvent removal was hydrated and homogenized by ultrasonication to obtain SIL-emulsomes, which were characterized by average particle diameter of about 364 nm and zeta potential of -34 mV. SIL-emulsomes exhibited sustained release *in vitro* and better pharmacokinetic parameters *in vivo* compared with SIL solution as control.

Regarding the application of NLCs to entrap efficiently SIL and thus increase its bioavailability and consequent therapeutic activity, the specific literature is plenty of various studies such as the one published by Jia et al. [76], who explored the potential of NLCs for the intravenous (i.v.) delivery of SIL. For the preparation of SIL-NLCs with the method of emulsion evaporation at a high temperature and solidification at a low temperature, GMS was used as solid-lipid ingredient while MCT was selected as liquid-lipid material. First, a nanoemulsion was obtained by adding dropwise a hot alcoholic solution of SIL, lipids (GMS and MCT) and lecithin, to an aqueous phase containing 1.5% pluronic F68 under mechanical stirring. Then, the nanoemulsion was quickly dispersed into cold distilled to promote the SIL-loaded NLC dispersion. The mean particle size was about 230 nm and zeta potential -20.7 mV. It was also observed that the drug entrapment efficiency and drug loading of nanoparticles increased from 72.31 to 96.87% and from 3.63 to 4.84%, respectively, with the increase of MCT % from 0 to 30 wt% [77]. From *in vitro* studies, a burst drug release was detected at the initial stage due to a fraction of SIL loaded into the liquid-lipid-enriched outer layers of NLCs. Afterwards, a more prolonged drug diffusion occurred when the SIL fraction dispersed into the nanoparticle core was gradually released by erosion of the lipid matrix. Moreover, SIL-NLCs showed higher AUC values and a prolonged residence time in the blood circulation compared with SIL solution as control.

More recent improvements in the formulation of SIL-NLCs consider several variants of preparation, such as hot HPH method [78], emulsification and ultrasonication method [79], and solvent diffusion followed by ultrasonication to develop SIL-NLCs gel for epidermal tissue deposition enhancement, [80]. Finally, SEDDS and NLCs were employed to enhance SIL oral bioavailability at level of gastrointestinal membrane and treat obesity-induced NAFLD [81].

3.2. Polymer-based delivery agents

In literature exists a variety of solutions designed by researchers to effectively encapsulate SIL in biocompatible and biodegradable polymeric nanosystems such as polymeric micelles, composites and solid nanodispersions. Altogether they represent a very efficient strategy by which a poorly

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water-soluble drug can be dispersed into an inert hydrophilic polymer matrix. As the polymeric erosion progresses, the loaded drug is released in the form of very fine particles for rapid dissolution.

3.2.1. Inclusion in polymeric matrices

The choice of the formulation method is fundamental to optimize the performance of the final product as described by Sonali et al. [82], who compared kneading, spray drying and co-precipitation techniques in the preparation of SIL-loaded solid dispersions using HPMC as a hydrophilic polymeric carrier. *In vitro* studies suggested the following enhancement in SIL dissolution compared to pure drug: co-precipitation (2.5 fold) > spray drying (1.9 fold) > kneading (1.5 fold).

A novel SIL-based formulation for the treatment of atopic dermatitis (AD) was designed by using pluronic-lecithin organogels, owing to their biphasic composition and versatility as transdermal and topical drug delivery systems [83]. The tested formulations contained 20% oil phase (lecithin / IPM) and 80% aqueous phase (pluronic). The high penetrating ability and hydration effect of the organogel base, provided a significant improvement in the signs and symptoms of AD patients.

Nguyen et al. [84] developed a high-payload supersaturating delivery system by preparing SIL-chitosan nanoparticles from a drug-polysaccharide complexation (nanoplex). At the optimal pH and chitosan-to-SIL charge ratio, the size and zeta potential of nanoplex were, respectively, 243 nm and 21 mV, while the complexation efficiency and yield were obtained in the range 83-87% and 55-63%, respectively. The nanoplex stability after either short or long-term storage and prolonged supersaturation in the presence of HPMC, demonstrated its feasibility as a new strategy for improving SIL bioavailability.

Liquid crystalline (LC) cubosomes coupled to P407 have been also formulated to enhance the oral bioavailability of SIL [85]. The LC matrix system was prepared by a melting/congealing method with GMO to P407 ratio of 100 : 12 at which cubic LC phases formed upon hydration. The amounts of drug dispersed as amorphous state in the matrices were fixed within the range 2-8%. SIL entrapped into the GMO-P407 LC system, manifested a 3.5-fold increase in bioavailability after oral administration as compared with a commercial drug formulation.

An increase of SIL aqueous solubility by almost 650-fold compared to bare drug powder was achieved by incorporating the drug into a solid dispersion, prepared by spray-drying SIL aqueous suspensions in presence of a surfactant-polymer mixture [86]. This strategy, beside using water instead of the organic solvent, led to a reduced ratio of hydrophilic polymeric carrier and drug in the final solid dispersion. A series of 1% aqueous solutions of biocompatible polymers and surfactants were tested to evaluate their capacity as SIL carriers in enhancing the aqueous solubility of the drug. PVP and Tween 80 provided the highest drug solubility of about 800 mg/ml and 2500 mg/ml, respectively, and were selected for preparing SIL-loaded solid dispersions. The optimised SIL/PVP/Tween 80 formulation (5 : 2.5 : 2.5, w/w/w), combined both relatively smaller amounts of carriers and increased drug solubility and dissolution. Compared to a commercial product, the proposed solid dispersion improved the oral bioavailability of the drug in rats by almost 3-fold and also exhibited advanced hepatoprotective bioactivity.

NPs formulated by loading SIL into the cationic copolymer Eudragit, were prepared by nanoprecipitation technique using PVA as a stabilizer to investigate their anticancer efficacy in oral carcinoma (KB) cells [87] and their ability to reverse the fibrosis-induced cholestasis in rats [88].

Another recent investigation reports the formulation of Eudragit-based SIL-loaded NPs, prepared by nanoprecipitation technique utilizing different PVA concentrations at various organic/aqueous phase ratios. The formulation of suitable particle size of about 85 nm, entrapment efficiency of 83.45% and *in vitro* 100% drug release after 12 h, was selected for *in vivo* hepatoprotective activity and toxicity studies [89].

One of the recently published research reports a formulation composed by SIL-PVP-PEG (0.25 : 1.5 : 1.5, on weight basis) in form of polymeric composite, which promotes an increase in the SIL solubility of more than 24 mg / ml and an excellent dissolution profile [90]. The enhancement in drug solubility and dissolution has been attributed to a better SIL wetting driven by the hydrophilic

polymers, complete conversion of the crystalline components into the amorphous state and molecular level homogeneousness of SIL, PVP and PEG in the resulting polymeric composite.

3.2.2. Dendrimers and polymeric NPs

SIL water solubility was significantly improved by embedding the drug in nano-containers made by dendritic macromolecules such as PAMAM [91]. In particular, both amine-terminated full generation (G2 and G3) and ester-terminated half-generation (G1.5 and G2.5) PAMAM dendrimers, were tested for their potential use as SIL solubility enhancers [92]. Low molecular weight drugs such as SIL, may be encapsulated into the inner core of PAMAM dendrimers or interact with their positively charged surface groups. The estimated number of SIL molecules incorporated into dendrimers ranged between 20 and 32 for G2 and G3 while for G1.5 and G2.5 it was comprised between 4 and 6. The analysis of pharmacokinetic experiments and oral bioavailability data demonstrated that drug-dendrimer complex could improve the oral absorption of SIL. One of the open question is the mechanism whereby PAMAM dendrimers increase the small intestinal absorption of the drug. Probably more than one mechanism, such as the effect of paracellular transport of drug-dendrimer complexes through the epithelium, improved contact with the epithelium itself and higher absorption through the endocytosis process, could contribute to the greater oral bioavailability of SIL vehicled by PAMAM dendrimers.

SIL solubilization into polymeric micelles was achieved using an amphiphilic derivative of the Carboxy-Methyl-Chitosan (CMCHS) synthetized by Sui et al. [93]. At the concentration of 10 mg / ml of polymer, the concentration of solubilized SIL increased more than 13 times that pure drug dissolved in water. SIL-loaded polymeric aggregates were found much bigger and polydisperse than blank micelles. *In vitro* tests showed a slow SIL release from micellar solution, lasted up to 40 h.

Polymeric PLGA nanoparticles, prepared by the single ESE technique, have been also used to solubilize SIL as described in ref. [94]. Then, the SIL-loaded PLGA NPs were encapsulated into a calcium-crosslinked alginate matrix to obtain a series of biodegradable pH-responsive hydrogel microparticles. The developed particles showed promising biodegradability and sustained SIL release profiles, as well as improving overall drug dissolution.

SIL NPs were formulated by ESE technique using poly-ε-caprolactone as a biodegradable polymer to achieve sustained release, improvement in bioavailability as well as enhancement of liver protection following intravenous administration [95]. The mean particle size and encapsulation efficiency ranged from 130 - 430 nm and 91 - 95 %, respectively, depending on polymer concentration. Increase in polymer content led also to delayed drug release due to increase in particle size. Both *in vitro* and *in vivo* tests suggested that the developed SIL NP formulation may be useful in the treatment of cirrhosis and fibrosis diseases.

Among various methods used in the preparation of chitosan nanoparticles, namely, microemulsion, emulsification/solvent diffusion, polyelectrolyte complex and ionic gelation, the latter was chosen by Pooja et al. [96], to prepare chitosan-TPP nanoparticles for oral delivery and to evaluate the potential of encapsulated SIL for anticancer activity. In this method, chitosan was dissolved in 1% (v/v) acetic acid and SIL dissolved in acetone was added drop wise to polymer solution, then TPP to allow spontaneous formation of nanoparticles. The optimal formulation was characterized by an entrapment efficiency of about 83 % with particle size of 264 nm (PDI < 0.3) and zeta potential of 37.4. SIL was amorphous and homogenously dispersed in polymeric matrix, and no chemical interactions between drug and chitosan were deduced from the analysis of FTIR spectra. Cytotoxicity studies, revealed that SIL entrapped in chitosan NPs was more effective than free drug.

In a different study, SIL NPs were prepared by Snima et al. [97] through ESE method using PLGA as polymeric nanocarrier. The particle hydrodynamic diameter was about 220 nm and SIL entrapment efficiency was 60%. *In vitro* tests showed a slow and sustained drug release profile at physiological conditions. Moreover, the NPs with desired drug release kinetics were capable of delivering SIL into prostate cancer cells to induce differential anticancer effect.

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Guhagarkar et al. [98] reported the design of PolyEthylene Sebacate (PES) NPs functionalized with the polysaccharide Pullulan (PUL) as hepatic targeting agent. SIL was entrapped into the NPs prepared by nanoprecipitation method giving rise to final the PES-SIL-PUL biodegradable polymeric nanocarrier. The entrapment efficiency was about 43% with mean particle size of 283 nm. The liver protection activity was ascertained in a model of induced hepatotoxicity in rats, by detecting the reduction in levels of serum transaminase. Histopathological evaluation of liver tissues also confirmed the enhanced hepatoprotection upon oral administration of PES-SIL-PUL NPs.

Another formulation of SIL-loaded NPs with particle size of about 100 nm, was proposed by Zhao et al. [99] by ESE technique and freeze-drying method, using P188 as polymer. SIL NPs absorption in the organs was significantly higher than that of drug suspension and very high SIL concentrations were observed in the liver with a long retention time.

Ma et al. [100] used the Bletilla striata polysaccharide (BSP) modified with stearic acid to encapsulate SIL into self-assembled spherical NPs with a mean diameter of 200 nm. Compared to the drug suspension, the developed formulation with SIL encapsulation efficiency of about 80%, was able to improve cytotoxicity and cell uptake in HepG2 cell lines *in vitro*.

In another biopolymer-based treatment, the incorporation of SIL in nanoparticles composed by water soluble chitosan and poly-γ-glutamic acid (γ-PGA), a natural anionic peptide produced by several *Bacillus* species, has been clearly revealed to be an effective approach for improving the drug solubility and its antimicrobial activity [101]. Hence, it was demonstrated that biodegradable films containing SIL NPs could efficiently control the growth of food microorganisms.

The hepatoprotective role of SIL entrapped into chitosan NPs (SIL-NPs) by ionotropic gelation method, and the anti-inflammatory effect of inulin nanoparticles (IN-NPs) synthesized using the emulsion method, were evaluated singly or in combination against hepatotoxicity induced by the mycotoxin Deoxynivalenol (DON) in vivo [102]. It has been shown that the combined treatment with SIL-NPs plus IN-NPs was able to overcome significantly the toxicity of DON in the liver.

3.3. Nanocrystals, nanosuspensions and nanohybrid DSs

Drug nanosuspensions are sub-micron colloidal dispersions of pure drug particles, which are stabilized by surfactants or polymeric steric stabilizers [103]. Nano- and micronization technologies improve the oral bioavailability and dissolution rates and prolong the half-life of sparingly soluble drugs. For instance, Zhang et al. [104] applied the ESD method to produce uniform SIL nanospheres with a mean size of ~240 nm as well as micronized rod-shaped and spherical particles obtained by controlling the temperature and SDS concentration. X-ray powder diffraction (XRPD) investigations demonstrated a low crystalline state for the rod-shaped smaller particles, which in turn manifested a better dissolution property than the larger spherical ones. However, although the precipitation technology is simple and cost effective, the tendency of the pharmaceutical particles to grow, and the difficulty in inhibiting that growth, posed obstacles to their production at industrial level.

Wang et al. [105] used the HPH technology to design SIL nanosuspension formulations for oral and i.v. administrations with different particle sizes. XRPD and differential scanning calorimetry (DSC) experiments showed that the crystalline structure of SIL was not perturbed as a result of the homogenization and freeze-drying processes. *In vitro* dissolution profiles and solubility tests of various nanosuspensions, including un-milled commercial SIL and a physical mixture for comparison, yielded an increase in solubility and dissolution rate following the reduction of particle size as predicted by the Noyes-Whitney equation [106]. Such nanosuspensions wre able to improve the permeability of the transport of SIL across the Caco-2 cell monolayer. *In vitro* results were further confirmed by the pharmacodynamics and tissue distribution studies in beagle dogs and mice [107].

An alternative methodology for the preparation of SIL-based nanodispersion with enhanced dissolution rate was performed by Cui et al., using a microchannel antisolvent precipitation combined with spray-drying [108]. This was the first work reporting SIL-loaded nanodispersion produced via microfluidics, enabling efficient control over the particle size, homogeneity, and drug release performance.

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The anticancer efficacy of SIL nanosuspensions was also tested by Zheng et al. [109], by carrying *in vitro* assays on human prostatic carcinoma PC-3 cell line. The HPH method has been recently used to test the feasibility of SIL nanocrystals as stabilizing agent of a Pickering emulsion of glyceryl monocaprylate oil droplets in water and provide a novel formulation free of any surfactants or polymer stabilizer [110]. The authors obtained flat spherical drug nanocrystals with mean particle size of 300 nm for homogenization pressure as high as 100 Mpa. Scanning electron micrographs supported a core-shell microstructure of the emulsion characterized by a core of oil saturated with SIL and a shell of SIL nanocrystals. Compared with SIL nanocrystalline suspension (SN-NCS), SIL nanocrystal self-stabilized Pickering emulsion (SN-SSPE) showed a better dissolution profile with a faster rate and more efficient dissolution. This difference was attributed to the fraction of drug dissolved in the oily phase of SN-SSPE, which could be released more easily than SN-NCS.

Yang et al. [111] applied the solution-enhanced dispersion approach by supercritical fluid (SEDS) technology using various polymeric excipients to produce SIL solid dispersions with improved dissolution and bioavailability of the active ingredient tested in rodents.

An example of nanohybrid materials for the advanced delivery of SIL in therapeutic applications is represented by SIL-loaded magnetite NPs (Fe_3O_4) modified with PLGA-PEG copolymers as reported by Ebrahimnezhad et al. [112], to investigate their inhibitory effect on Telomerase expression in T47D human breast cancer cell line. The PLGA-PEG-Fe_3O_4 NPs with a SIL loading capacity of about 76%, besides being biocompatible, possessed the advantage of being directed into the target tissue by the action of an external magnetic field [113]. The cytotoxic effect on the T47D cell line increased with increasing the concentration of SIL-loaded NPs, demonstrating the feasibility of this nanodrug in down-regulation of Telomerase gene expression in cancer cells.

In a more recent formulation, Fe_3O_4 NPs were coated with chitosan by the coprecipitation method and then loaded with SIL [114]. The capability to act simultaneously as drug nanocarrier and magnetic resonance imaging (MRI) contrast agent was tested through various methods and techniques. The zeta potential of bare magnetite NPs in aqueous dispersion changed from negative (-24.2 mV) to positive (+31.6 mV) values upon coating with chitosan, demonstrating the presence of terminal amino groups on the particle surface. The average particle size was about 18 nm with SIL entrapment efficiency of 95%. *In vitro* studies revealed a sustained drug release pattern.

Another interesting nanohybrid systems containing stimuli-sensitive components was reported by Fazio et al. [115], where SIL and gold nanocolloids synthesized by laser ablation were co-loaded into the PLGA-PEG copolymer in a single step procedure. The SIL-loaded PEG-PLGA-Au nanocomposite with a polymer/drug weight ratio of 50 : 5, was prepared by a modified emulsion-diffusion method. The hybrid NPs allowed SIL to be released in a controllable manner upon thermal activation of Au NPs incorporated in the polymer matrix, stimulated by irradiation of a red laser source of low power density (21 mW cm^{-2}), partially transparent to human flesh. The localized and intensive heating of Au NPs causes the thermal expansion of the polymer, with the consequent release of drug that starts to diffuse out. As a potential application of this nanocomposite, it was suggested the use of wirelessly controlled nanowires responding to an electromagnetic field generated by a separate device. This engineering system would eliminate the tubes and cables required by other implantable devices with the risk of infections and other complications, and activate the release of the drug near areas of the body that are often difficult to reach.

3.4. Nanostructured materials based on inorganic compounds

Inorganic nanomaterials are currently considered as powerful and very efficient drug carriers due to their versatile nanostructure, functional properties and controlled drug release behaviors. Moreover, they can exhibit excellent biocompatibility, biodegradation, in vivo stability, low cytotoxicity and nonimmunogenic profiles, thereby making these nanovectors an ideal candidate for oral and parenteral drug delivery [116]. One of the first applications appeared in the literature on the exploitation of these inorganic nanomaterials as carriers of the poorly soluble SIL, in order to improve its bioavailability was provided by Cao et al. by engineering porous silica nanoparticles (PSNs) [117-

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119]. They were prepared using a W/O microemulsion for the synthesis of monodispersed nonporous silica nanoparticles to form cores and an ultrasonic corrosion method to create regular nanometer-sized pores with sodium carbonate solution. SIL-loaded PSNs were obtained by immersing the porous NPs in SIL ethanol solution and stirring for 24 h, and after various treatments the entity of drug entrapment in PSN was quantified in about 69%. The mean hydrodynamic particle diameter was 56.2 nm, characterized by a unimodal and narrow size distribution. *In vivo* studies indicated multistage release pattern after oral administration of SIL-loaded PSNs, where an initial delay in the rate of drug absorption was followed by an enhanced extent of absorption with a high plasma concentration maintained even up to 72 h. Those results suggested that the PSNs could be used as a promising SIL nanovectors for sustained-release systems, satisfying the need for prolonged treatment after oral administration.

Commercially available carboxylated multiwalled carbon nanotubes (COOH-MWCNTs) [120], have been employed by Tan et al. [121] to covalently conjugate SIL for the advanced drug delivery in therapeutic applications. Drug release from the carbon nanotubes showed a sustained- and pH dependent behaviour while a significant *in vitro* cytotoxicity was expressed against two human cancer cell lines at lower concentrations of 1.56-6.25 μg/mL when compared to free drug alone.

Other types of inorganic NPs, such as amorphous calcium phosphate (ACP) nanospheres and crystalline hydroxyapatite (HAP) nanorods, which enjoy favourable chemical properties similar to the inorganic constituents of natural bone tissue, were exploited by Chen et al. to encapsulate SIL [122]. SIL loading into ACP and HAP was achieved by immersion of both types of NPs, previously synthesized by polymeric micelle-templated technique, in SIL-containing ethanol solutions. Both ACP nanospheres and HAP nanorods manifested relatively high drug loading capacity of 900 and 825 mg g^{-1}, respectively. The drug release in both simulated intestinal (SIF) and gastric (SGF) fluids of ACP and HAP delivery systems, exhibited a rapid SIL release at the early stage (< 2h), followed by a slow and sustained release in a period of about 17h.

Finally, a novel micelle-templated synthesis of porous calcium phosphate microparticles was developed by Zhu et al. [123], to improve the delivery of the poorly water-soluble SIL *in vitro* and *in vivo*. In particular, a mixed micellar system composed by PVP/SC/phospholipid in suitable mass ratios, was adopted innovatively as a new type of template to fabricate the calcium phosphate micron-sized carriers. The drug encapsulation was proven to be incorporated into the porous structure of microparticles after the removal of the micellar template, thus leading to prolonged release *in vitro* and enhanced absorption *in vivo*. An excellent linear relationship was obtained between *in vitro* dissolution and *in vivo* absorption data, recorded in two media at different pH. This correlation could suggest the possibility to predict *in vivo* pharmacokinetic behavior through the observed *in vitro* release profiles.

3.5. Cyclodextrin inclusion complexes

Natural cyclodextrines (CDs) are widely used in pharmaceuticals, drug delivery systems, cosmetics, food technology and chemical industries. They can be found in commercially available medications, including tablets, eye drops, and ointments (see ref. [124] for a recent review on the subject). Formulations based on SIL inclusion complex with β-CD were reported by Ghosh et al. [125]. They were prepared by different methods, such as, physical mixing, kneading, co-precipitation and solvent evaporation. The inclusion complex prepared by the co-precipitation method led to best results regarding the drug sustained release performance. In another investigation, a lyophilized SIL-HP-β-CD complex was prepared and evaluated *in vitro* by Kellici et al. [126], who performed detailed physicochemical studies on the SIL-CD interactions at the molecular level and testes the respective bioavailability on MCF-7 cancer cells. In a different study, SIL inclusion complexes with, respectively, HP-β-CD and RAMEB were developed in order to improve SIL anti-fibrotic activity at a lower therapeutical dose of 50 mg/kg, by increasing their potential solubilization and to prevent their metabolic degradation within the GIT after oral administration, [127].

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4. Conclusion and outlook

The appearance of increasingly advanced and performing Silymarin-based formulations has logically followed step by step the evolution of the nanotechnologies and nanosystems applied to the delivery of poorly water-insoluble drugs and active principle ingredients. In the Table 1 the types of nanocarries reviewed in the previous sections have been systematically collected and linked to their respective methods of preparation, reported bioactivity, route of administration and related bibliographic sources. We hope that this study could represent a useful reference for a broad and updated overview on the most efficient and relevant nanotechnologies aimed ultimately at improving the therapeutic efficiency of Silymarin.

Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

Type of Nanocarrier	Method of preparation	Bioactivity reported	Route of administration	reference
PC-BS Mixed micelles	TFD	Bioavailability studies	*In vitro*, oral	[31-33]
Lipid NPs	TFD	Inhibition of lung/ breast metastases	*In vitro*, i.p.	[34]
Microemulsion	aqu. titration	Dermal delivery system	topical	[35]
Emulsion / SEDDS	aqu. titration	Bioavailability / radioprotective	*In vitro* / oral	[36 ,39-43]
Nano-emulsion	aqu. titration / SPG / HPH	Bioavailability / hepatoprotective	*In vitro* / oral	[23, 44-46, 48]
Liposomes	ethanol injection / RPE / TFD / SEDS	Bioavailability / hepatoprotective / anti-inflammatory / anti-viral / uptake in Huh7.5	*In vitro* / oral / i.v. / buccal mucosa	[50-52, 59-60, 64-66]
Pro-Liposomes	TFD	Bioavailability / antioxidant / anti-cancer	*In vitro* / oral	[53, 56[a]]
PEG-Liposomes	TFD	Bioavailability / anti-cancer	*In vitro* / oral	[57-58]
SLN *vs* NLN	hot HPH	Bioavailability	*In vitro* / oral	[71]
SLNs	hot (cold) HPH/ EES / TFD	Bioavailability / hepatoprotective	*In vitro* / oral / i.v.	[72-75]
NLCs	ESE / hot HPH / emulsific. & ultrason. / solv. diff. / ultrason.	Bioavailability / tissue distribution studies / treatment of obesity-induced NAFLD	*In vitro* / oral / i.v.	[76-81]
Polymeric organogel / cubosomes	mix org./aqu. phases	treatment of atopic dermatitis	*In vitro* / topical	[83,85]
nanoplex	Chitosan complexation	Bioavailability studies	*In vitro*	[84]
Solid dispersion / polymeric composite	spray-drying / nanoprecipitation / solvent-evaporation	Bioavailability / hepatoprotective / anticancer in oral carcinoma	*In vitro* / oral	[86-90]

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PAMAM dendrimers	complexation	Bioavailability studies	*In vitro* / oral	[92]
Polymeric micelles	ESE / nanoprecipitation / polyelectrolyte complex-ionic gelation	Bioavailability & cytotoxicity studies / hepatoprotective / uptake in HepG2 cells	*In vitro* / oral	[93-102]
Nanosuspensions / nanocrystals	ESD / HPH / SEDS / microfluidics	Bioavailability & tissue distribution studies / prostatic carcinoma	*In vitro* / oral	[104-105, 107-111]
nanohybrid materials	Coprecipitation / PLGA-PEG-Fe_3O_4 Chitosan-Fe_3O_4 PEG-PLGA-Au	down-regulation of Telomerase gene expression in breast cancer cells	*In vitro* / oral	[112, 114-115]
Porous silica NPs	W/O microemulsion	Bioavailability studies	*In vitro* / oral	[117-119]
carbon nanotubes	covalent conjugation	cytotoxicity studies / anticancer	*In vitro*	[121]
Ca-phosphate / hydroxyapatite	Polymeric micelle-template	Bioavailability studies	*In vitro*	[122-123]
β-CD inclusion complex	coprecipitation	Bioavailability on MCF-7 cancer cells / anti-fibrotic activity	*In vitro* / oral	[125-127]

[a] The encapsulated drug is 2,3-dehydrosilymarin, an oxidized form of SIL

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List of abbreviations:

AUC = area under the plasma drug concentration-t curve;
β-CD = β-cyclodextrin;
Brij 78 = polyoxyethylene 20 stearyl ether;
BS = Bile Salts;
C_{max} = maximum plasma drug concentration;
Capryol 90 = propylene glycol monocaprylate;
CMCHS = carboxymethylchitosan;
CHOL = cholesterol;
Cremophor EL = polyoxy-35-castor oil;
Cremophor RH40 = polyoxyl 40 hydrogenated castor oil;
DCP = dicetylphosphate;
D-GaIN = D-galactosamine;
DPPC = DiPalmitoylPhosphatidylCholine;
DSPE = DiStearoylPhosphatidylEthanolamine;
EES = Emulsification Evaporation Solidification;
ESD = Emulsion Solvent Diffusion;
ESE = Emulsion Solvent Evaporation;
EVO = extra virgin olive;
GA = glycyrrhizic acid;
GIT = gastrointestinal tract;
GMO = GlycerylMonoOleate;
GMS = GlycerylMonoStearate;
HCO-X® = PEG-X Hydrogenated Castor Oil, (X = 40, 50);
HP-β-CD = 2-hydroxypropyl-β-cyclodextrin;
HPH = High Pressure Homogenization;
HPMC (E50LV) = HydroxyPropyl MethylCellulose;
HSPC = Soya Hydrogenated L-α-PhosphatidylCholine;
i.p. = intraperitoneal;
IPM (Estol) = isopropyl myristate;
isoSIL = isosilybin;
i.v. = intravenous;
Labrafac® CC = Medium Chain Triglycerides (MCT);
Labrafil® = transesterified ethoxylated vegetable oils;
Labrasol® = caprylocaproyl polyoxylglycerides (macrogolglycerides);
MCT = Medium chain triglycerides;
NAFLD = NonAlcoholic Fatty Liver Disease;
NLCs = Nanostructured Lipid Carriers;
NPs = nanoparticles;
OA = oleic acid;
P188 = Poloxamer 188;
P407 = Poloxamer 407;

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PAMAM = polyamidoamine:
PC = L-α-PhosphatidylCholine;
PDI = polydispersity index;
PEG = polyethyleneglycol;
PLGA = poly(D,L-lactic-co-glycolic acid);
PPC = Polyene PhosphatidylCholine;
PVA = Polyvinyl alcohol;
PVP = polyvinylpyrrolidone;
RAMEB = randomly methylated-β-cyclodextrin;
RPE = reverse phase evaporation;
SA = stearyl amine;
SC = Sodium Cholate;
SCF-CO_2 = SuperCritical Fluid of carbon dioxide;
SDC = Sodium DeoxyCholate;
SEDS = Solution-Enhanced Dispersion Supercritical fluids;
SEDDS = Self Emulsifying Drug Delivery System;
Sefsol 218 = propylene glycol monocaprylic ester;
SGC = Sodium GlycoCholate;
SGF / SIF = simulated gastric fluid (pH 1.2) / simulated intestinal fluid (pH 7.4)
SIL = Silybin or silybinin or silymarin extract;
SILcr = silycristin;
SILdi = silydianin;
Sito-G = β-sitosterol β-D-glucoside;
SLNs = Solid Lipid Nanoparticles;
Solutol® HS 15 = PEG (15)-hydroxystearate;
SPC = Soya L-α-PhosphatidylCholine;
SPG = Shirasu Porous Glass membrane emulsification;
SPMM = Na cholate/phospholipid mixed micelles;
STC = Sodium TauroCholate;
SUV = small unilamellar vesicles;
TFD = Thin-Film Dispersion;
TNF-α = Tumour Necrosis Factor-α;
TPGS = D-α-Tocopheryl PEG 1000 Succinate;
TPP = TriPolyPhosphate;
Transcutol® = diethylene glycol monoethyl ether;
Triacetin = glycerol triacetate;
Tween 20 = polyoxyethylene sorbitan monolaurate (polysorbate 20);
Tween 80 = polyoxyethylene sorbitan monooleate (polysorbate 80);
TXF = taxifolin;

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