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***Nano-drug delivery systems:
a useful approach to improve
the biopharmaceutical properties
and the pharmacological potential
of plant-derived molecules***

Settore Scientifico Disciplinare CHIM/09

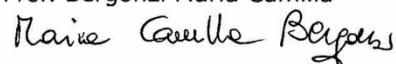
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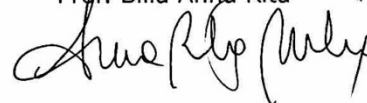
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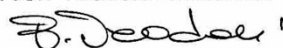
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ABSTRACT

Recently, the use of herbal drugs has been increased all over the world due to their therapeutic effects and fewer adverse effects as compared to conventional medicines. However, many herbal drugs and herbal extracts demonstrate low *in vivo* activity due to their poor solubility, improper molecular size or high metabolism, scarce absorption and bioavailability.

Novel drug delivery systems open the door towards the development of enhancing bioavailability of plant-derived molecules. The innovative formulations are reported to have remarkable advantages over conventional formulations of plant actives and extracts which include increase of solubility, bioavailability, protection from toxicity, enhancement of pharmacological activity and intracellular uptake, modification of pharmacokinetics and biodistribution, improved tissue macrophages distribution, sustained delivery and protection from physical and chemical degradation. There are numerous examples of the application of the nano-sized drug delivery systems in this field related to isolated compounds or purified extracts.

This research concerns the improvement of the biopharmaceutical properties and the pharmacological potential of plant-derived molecules.

After the optimization of analytical methods for qualitative and quantitative characterization of the compounds, this work was focused on the development of different nano-drug delivery systems, such as micro- or nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, liposomes, and albumin nanoparticles, for oral and brain delivery of herbal extracts or single molecules.

The obtained formulations were physically and chemically characterized by Light Scattering techniques, transmission electron microscopy observations, high performance liquid chromatography, spectrophotometric and calorimetric analyses.

All the developed nanocarriers enhanced the solubility of the bioactives when compared with their aqueous suspensions.

Chemical and physical stability during storage, in simulated gastrointestinal environment, in the presence of human serum albumin and in rat plasma was tested.

The dialysis bag method was employed to evaluate the release of the entrapped molecules in different pH conditions.

In vitro permeation, cytotoxicity and uptake studies using different models such as parallel artificial membrane permeability assay (PAMPA), Caco-2 cell line, hCMEC/D3 cells were performed. These *in vitro* studies could provide useful information about dissolution and permeation/absorption aspects, which further could be correlated to *in vivo* bioavailability studies.

Furthermore, the mucoadhesive properties of chitosan-coated formulations were investigated *in vitro*, by the turbidimetric method and the evaluation of the ζ -potential and performing *ex-vivo* experiments using the rabbit nasal mucosa in a Franz-type diffusion apparatus.

Besides, to evaluate the ability of the delivery systems to cross the blood-brain barrier and reach brain tissues, *in vivo* distribution and fate after intravenous administration in healthy rats were studied.

Finally, nanostructured lipid carriers designed with silymarin as a payload were studied in a diabetic mouse model as one alternative/complementary remedial in systemic hyperglycemia, hypertriglyceridemia, and neuropathy.

Thus, the present research proposed novel and promising strategies based on the development of nano-drug delivery systems to enhance the biopharmaceutical properties of plant active compounds and improve their performance in therapy.

INTRODUCTION

Natural products from plants, animals, and minerals have played a key role in treating and preventing human diseases since ancient times and they still represent a significant source of modern drugs. Currently, the global market of natural products, which is mainly derived from the plant kingdom, is estimated at more than US\$80 billion and it continues to grow. Herbal drugs and extracts sold as dietary supplements, food, or herbal medicinal products are the principal products, while single isolated constituents represent a lesser proportion. Besides, according to the WHO, between 65 and 80% of populations in developing countries presently use medicinal plants as therapeutic remedies (Cameron et al., 2011).

Indeed, natural products still represent a primary source of drugs thanks to their enormous structural and chemical diversity to which synthetic libraries cannot compare (Beghyn et al., 2008).

Over time, drug discovery has undergone many transformations, and in the last decade, it has become clear that the use of drugs targeting single sites have low therapeutic value against multifactorial and complex diseases, especially cancer and diabetes (Lu et al., 2012). By contrast, diverse natural products can modulate multiple targets activating various signalling or functional pathways (Morphy, 2010).

The tangible importance of natural products to the drug discovery process and their possible role in therapy is unquestionable. Conversely, their efficacy is frequently scarce because of low hydrophilicity and an intrinsic dissolution rate, and/or physical or chemical instability. Besides, they can have low absorption, limited biodistribution, first-pass metabolism, poor penetration and accumulation in the organs of the body, or trivial targeting efficacy.

Without a doubt, extracts have a generally better therapeutic performance than single constituents. Occasionally, the constituents provide synergistic or simply additive action resulting in an enhanced therapeutic value. Conversely, extracts are usually very complex mixtures, made up of molecules with different solubility and chemical structures. They are generally considered poor drug candidates because they commonly need repeated administrations or higher doses with respect to the single constituents. Accordingly, pure constituents are easier to handle, even if they are more expensive to produce because of the isolation steps. The development of semisynthetic compounds or synthetic analogues or the production of prodrugs represents a further strategy to optimize the performance of natural products, even if, as in many cases, these approaches were not satisfactory (Kesarwani et al., 2013; Mehta et al., 2015).

The design and production of appropriate nano-drug delivery systems offers an additional and more advanced approach to optimized bioavailability and/or stability of isolated constituents and extracts. The prefix nano- comes from the ancient Greek *νᾶνος* through the Latin *nanus* meaning literally “dwarf” and, by extension, “very small”, namely between 50 and 300 nm. Numerous nanosized drug delivery systems have already entered into clinical use. The most pressing challenge for the near future is the design of multifunctional, structured materials able to target specific tissues or organs or containing functionalities to allow transport across biological barriers. These delivery systems are smartly designed to tag a variety of chemical, molecular, and biological entities. A successful drug carrier system needs to demonstrate optimal drug loading and release properties, a long shelf life, and exert a much higher therapeutic efficacy as well as lower side effects (Couvreur & Vauthier, 2006).

The promising therapeutic activities of many isolated natural products or extracts have encouraged nanotechnologists to design and formulate useful nanoformulations to improve solubility, stability, cellular uptake/internalization efficacy, specificity, tolerability, and therapeutic index. Currently, nanotechnology has an enormous impact on the medical field, significantly improving the performance of drugs concerning efficacy, safety, and patient compliance (Bilia et al., 2017).

Oral drug delivery, owing to its non-invasiveness and high patient compliance, is reputed first choice route for drug administration. Besides, the large surface area for absorption in the intestine along with the presence of M cells in various parts of the intestine provides a high incentive for the delivery of therapeutic drugs orally. However, despite the advantages, drug delivery through the oral route faces many challenges. These include rapid drug degradation in the gastrointestinal tract due to the acidic environment and proteolytic enzymes in the stomach as well as a physiological barrier for absorption due to the presence of tight junctions in the intestine. Moreover, the physical and chemical characteristics of a drug molecule may make it unsuitable for oral delivery such as low aqueous solubility/stability that can by itself contribute to low oral bioavailability. The nano-sized particles provide a vehicle for controlled or sustained release of drugs and a larger contact surface area for adherence to the intestine that can improve absorption. Furthermore, nanoparticles can be produced from a wide variety of materials to modulate their physico-chemical characteristics and their surfaces can be modified in various ways to improve enteric stability or the residence time through mucoadhesion. Consequently, size, shape, charge, surface chemistry and hydrophobic/ hydrophilic composition play an astonishing role in the ultimate success of the nanocarriers, optimizing bioefficacy.

The absorption of orally administered drugs is restricted to either transport through the cell (transcellular pathway) or between adjacent cells (paracellular pathway) (Figure 1).

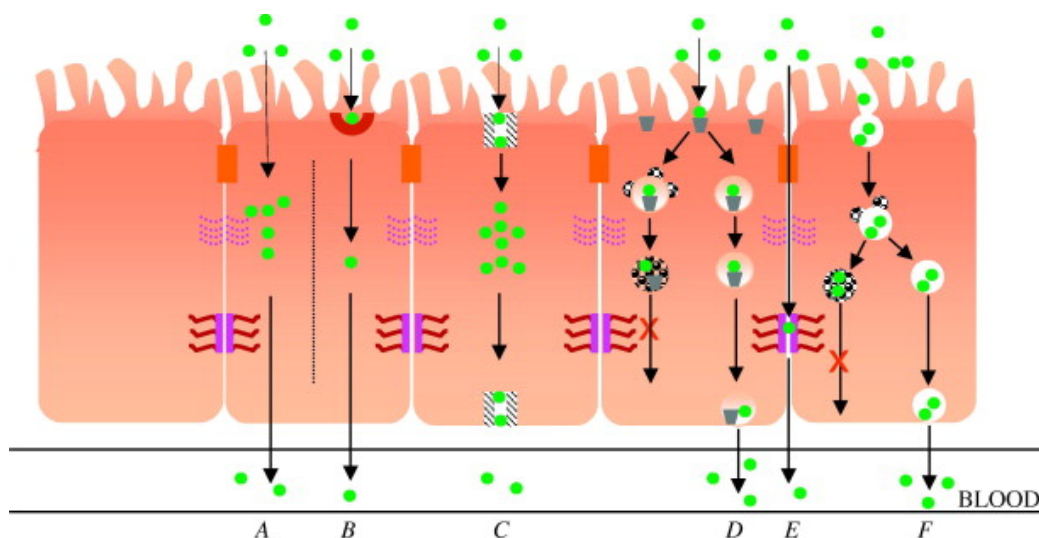


Figure 1. Schematic representation of the mechanisms involved in the absorption of xenobiotics in the small intestine. (A) Transcellular transport; (B) active transport; (C) facilitated diffusion; (D) receptor-mediated endocytosis; (E) paracellular transport; (F) pinocytosis.

(From: *Polymeric micelles for oral drug delivery* (2010). DOI: 10.1016/j.ejpb.2010.06.007)

Drugs absorbed by the transcellular pathway are generally low-molecular weight hydrophobic entities which are able to diffuse through the membrane, either on their own or associated with a specific membrane transporter. In both instances, the rate is determined by the concentration gradient across the intestinal membrane, with the blood acting as a sink. In some particular instances, drugs may be absorbed by fluid-phase endocytosis (pinocytosis), an energy-dependent saturable process in which the molecule travels inside membrane vesicles. However, once inside the cell, a fraction of these vesicles may fuse with enzyme-rich lysosomes leading to the degradation of the drug. Alternatively, the endocytosis of the drug may be triggered by its binding to a receptor, given sufficient structural analogy with the natural substrate. As with pinocytosis, this pathway may lead to the degradation of the absorbed substances.

Typically, hydrophilic molecules cannot freely diffuse through the intestinal membrane, due to their low affinity for the lipidic constituents. Therefore, in the absence of an appropriate membrane transporter, the paracellular route is the only one available for their absorption. In the paracellular space, the presence of junctions restricts the passage of large molecules. These include the tight junction, a protein complex that establishes links between adjacent cells through the intercellular space. Two other protein complexes, namely the adherens junctions and desmosomes further tighten cell association, without fusing cell membranes (Gaucher et al., 2010).

Nanocarriers with particles ranging in size between 40 and 50 nm have a maximum uptake *in vitro*, while a general range of 200-300 nm has been established for *in vivo* applications. In order to increase the stability, the intestinal absorption and, consequently, the bioavailability of the entrapped molecules, surface modifications can be achieved by surface coating with hydrophilic, stabilizing, mucoadhesive polymers/surfactants or by the production of biodegradable copolymers with hydrophilic segments. These modifications are related to changing of the ζ -potential, hydrophobicity, stability, mucoadhesive properties and protein adsorption on their surface (Bilia et al., 2017).

Besides, the delivery and release of natural active compounds into the brain is a challenging topic. The blood-brain barrier formed by the brain microvascular system is a membrane that separates blood from the extracellular fluid of the brain in the central nervous system. It is responsible for maintaining homeostasis of the brain by regulating the chemical environment, immune cell transport, and the entry of xenobiotics. The concentrations of water, ions, amino acids, hormones, and neurotransmitters in the blood undergo fluctuations, particularly after eating or exercise. If such fluctuations were allowed to occur in the brain, it would lead to local disruption of signal propagation and uncontrolled neural activity, and hence transport from the capillary lumen to the brain parenchyma must be tightly regulated. Immune cell transport (e.g., leukocytes) must be regulated as the brain is contained in a fixed volume in the skull and an inflammatory response could lead to an increase in intracranial pressure or cerebral oedema. Finally, the entry of toxins and pathogens, such as bacteria and viruses circulating in the blood, can lead to neuron cell death and hence must be prevented. The blood-brain barrier represents the most critical factor limiting the development of drugs for the central nervous system and it is characterized by relatively impermeable endothelial cells with tight junctions, enzymatic activity and active efflux transport systems. Brain microvascular endothelial cells constitute the anatomical basis of the blood-brain barrier and reduce the diffusion of molecules across the vessels. The tight junctions formed by brain microvascular endothelial cells regulate paracellular transport whereas transcellular transport is regulated by specialized transporters, pumps, and receptors. Several carrier or transport systems, enzymes and receptors that control the penetration of molecules in the blood-brain barrier endothelium have been identified.

Most of the strategies described for the delivery of actives across the blood-brain barrier can be enhanced by nanotechnology including open the tight junctions between endothelial cells, transcytosis through the endothelial cell layer, endocytosis by endothelial cells releasing the drug inside the cell. Furthermore, coating agents such as polysorbates inhibit the transmembrane efflux systems (i.e., P-glycoprotein) (Fonseca-Santos et al., 2015). Various novel drug delivery systems such as liposomes, microspheres, polymeric nanoparticles, lipid nanoparticles, inorganic systems have been proposed to overcome the limitations imposed by the blood-brain barrier (Figure 2).

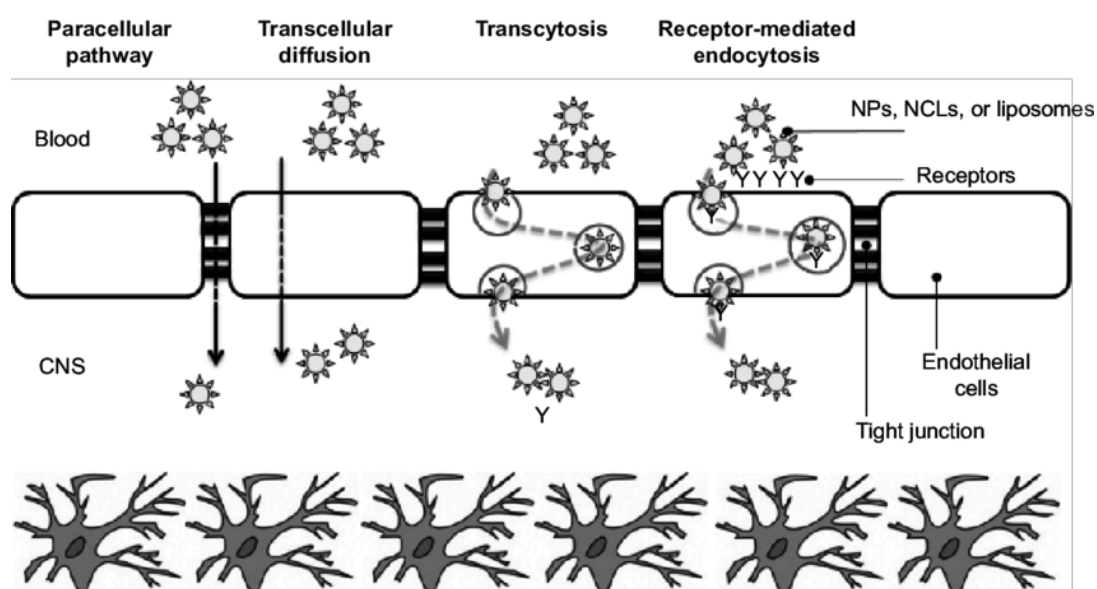


Figure 2. Main pathways for nanosystems to cross the blood–brain barrier to target to brain. (From: *Nanotechnology-based drug delivery systems for the treatment of Alzheimer’s disease* (2015). DOI: 10.2147/IJN.S87148)

Polymer-based carriers including nanoparticles (nanocapsules or nanospheres), dendrimers, micelles, lipid-based drug delivery systems such as solid lipid nanoparticles, nanostructured lipid carriers, microemulsions/nanoemulsions, nanocochleates and vesicular systems (liposomes and niosomes) and cyclodextrin complexes represent successful examples of nanoformulations loaded with bioactives (Sinico et al., 2005; Lai et al., 2007; Ajazuddin & Saraf, 2010; Rogerio et al., 2010; Chessa et al., 2011; Marianecchi et al., 2012; Puglia et al., 2012; Caddeo et al., 2013; Esposito et al., 2014; Pinho et al., 2014; Puglia et al., 2014; Righeschi et al., 2014; Parveen et al., 2015; Righeschi et al., 2016; Armendáriz-Barragán et al., 2016; Esposito et al., 2016; Falconieri et al., 2016; Leto et al., 2016; Offerta et al., 2016; Sinico et al., 2016; Anantaworasakul & Okonogi, 2017; Asprea et al., 2017; Grossi et al., 2017; Guccione et al., 2017; Nunes et al., 2017; Schlich et al., 2018).

Briefly, it is reported the description of the cited nano-drug delivery systems.

Polymeric nanoparticles

Nanoparticles are made of natural, semi-synthetic or synthetic polymers and depending upon the method of preparation, nanospheres or nanocapsules can be obtained and the drug should be dissolved, entrapped, encapsulated or attached to the nanoparticle matrix (Figure 3). Nanospheres are matrix systems in which the drug is physically and uniformly dispersed, while nanocapsules are the system in which the drug is confined to a cavity surrounded by a single polymeric membrane (Bilia et al., 2017).

Selection of polymeric materials is based on several desirable properties including biocompatibility, biodegradability, surface modification, and the propensity of functionalization for the development of new and safe carriers with controlled and targeted drug delivery concept.

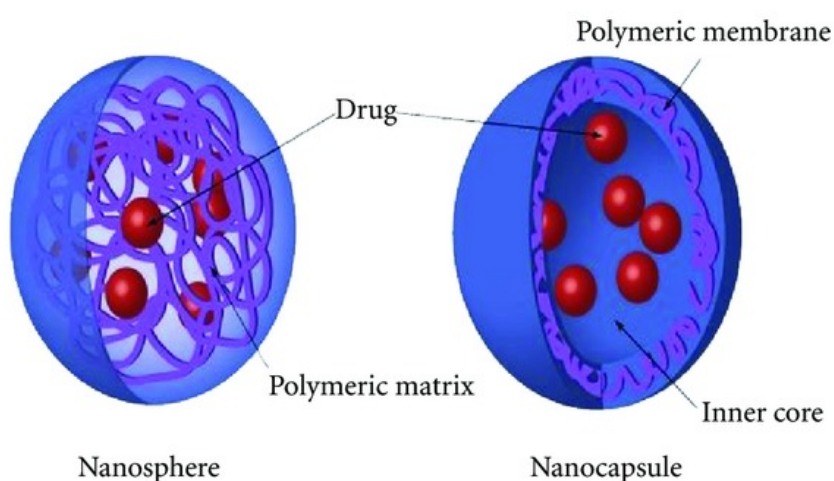


Figure 3. The two main types of polymeric nanoparticles with different drug-loading modalities. (From: Nanomedicine Future Medicine Ltd (2010)).

Several methods have been developed during the last two decades for preparation of nanoparticles, these techniques are classified according to whether the particle formation involves a polymerization reaction or nanoparticles form directly from a macromolecule or preformed polymer or ionic gelation method (Rao & Geckeler, 2011).

Polymers used for the nanoparticles are classified as synthetic or natural sources and biodegradable or non-biodegradable ones (Couvreur et al., 1995; Soppimath et al., 2001; Hans & Lowman, 2002). Natural polymers or biopolymers may be naturally occurring materials, which are formed in nature during the life cycles of green plants, animals, bacteria and fungi, and consist of polysaccharides and proteins. Polysaccharides include compounds from plant origin (e.g., pectin, cellulose and its derivatives, starch and its derivatives, arabic gum, carrageenan, and alginate) and polysaccharides from microbial or animal origin (e.g., xanthan gum, gellan gum and chitosan). NPs made of polysaccharides, due to their unique properties, are promising carriers to deliver and protect the physiological properties of hydrophilic drugs and have been successfully applied as drug delivery systems. As natural biomaterials, polysaccharides are stable, safe, non-toxic, hydrophilic, and biodegradable (Tonnesen & Karlsen, 2002; Le Corre et al., 2010; Moon et al., 2011; Charreau et al., 2013; Li et al., 2014; Osmalek et al., 2014; Hu et al., 2015; Kim et al., 2015; Chandra Hembram et al., 2016; Desai, 2016; Jana et al., 2016; Kumar et al., 2016; Li et al., 2017; Mohammed et al., 2017; Wang et al., 2018; Kaur et al., 2018). In addition to polysaccharides, several natural proteins are very useful to produce nanoparticles and they include gelatin, casein, albumin and soy proteins hydrolysate (Bhushan et al., 2017; Elzoghby et al., 2011; Foox & Zilberman, 2015; Bergonzi et al., 2016; Penalva et al., 2018; Tan & Ho, 2018).

Among the synthetic polymers, poly- α -cyanoacrylate alkyl esters (PCA), polyvinyl alcohol (PVA), polylactic acid (PLA), polyglycolic acid (PGA), and polylactic glycolic acid (PLGA), poly(N-vinyl pyrrolidone) (PVP), are the most used. Co-polymers are generally di-block, tri-block, multi-block or radial block copolymer structures (Grottgau et al., 2013; Hickey et al., 2015; Ahlawat et al., 2018).

Dendrimers

Dendrimers are well-defined hyperbranched polymers first developed under the name of cascade polymers (Figure 4). On a molecular level the dendritic branching results in semiglobular to globular structures, mostly with a high density of functionalities on the surface together with a small molecular "volume."

The name comes from the Greek word δένδρον (dendron), which translates to "tree". A dendrimer is typically symmetric around the core and often adopts a spherical three-dimensional morphology consisting of a series of branches around an inner core, molecular diameters are generally less than 10 nm, but can be modulated by varying dendrimer generations (G0, G1, G2, G3, etc.). Three different topological parts: (a) a central core which is either a single atom or an atomic group having at least two identical chemical functions; (b) building blocks with several interior layers composed of repeating units; (c) multiple peripheral functional groups, generally located on the exterior of the macromolecule, which play a key role in dendrimer properties.

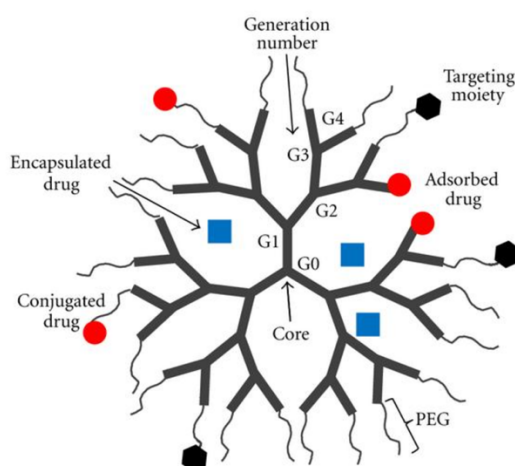


Figure 4. Combination drug delivery systems based on dendrimers: concurrent delivery of water-soluble and -insoluble drugs by adsorption to the surface, encapsulation within hydrophobic microcavities inside branching clefts or direct covalent conjugation to the surface functional groups.

(From: *Combination Drug Delivery Approaches in Metastatic Breast Cancer* (2012). DOI: 10.1155/2012/915375)

Dendrimers are synthesized from monomers using either convergent or divergent step-growth polymerization and are usually made from AB_n type monomers, each layer or generation of branching units doubling or tripling (n_2 , n_3) the number of peripheral functional groups. Drug molecules can be loaded either in the interior or can be adsorbed or attached to the surface groups. Different types of polymers such as polyamidoamine (PAMAM), poly(L-glutamic acid), polyethyleneimine, polypropyleneimine, and polyethylene glycol are used to fabricate dendrimers. They offer enormous advantages such as nanometric size range, ease of modification by modifying their termini, ease of preparation, and availability of multiple copies of surface groups for biological reorganization processes (Noriega-Luna et al., 2014; Wu et al., 2015).

Polymeric micelles

Polymeric micelles are special nanocarriers with a diameter smaller than 100 nm made of block-copolymers that consist of hydrophobic and hydrophilic monomer units dispersed in an aqueous media (Figure 5).

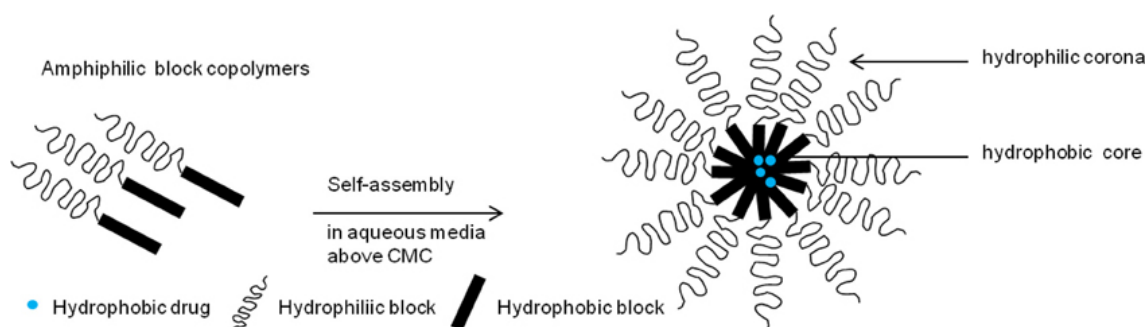


Figure 5. Micelle formation. Drug-loaded polymeric micelle formed from self-assembly of amphiphilic block copolymers in aqueous media.

(From: *Multifunctional polymeric micelles for delivery of drugs and siRNA* (2014). DOI: 10.3389/fphar.2014.00077)

Two monomers with different hydrophobicity can be conjugated and form a core-shell micelle structure, where the hydrophilic and hydrophobic blocks form the micelle shell and core, respectively, as in the case of Pluronic (polyoxyethylene polyoxypropylene block copolymers) that self-associate in aqueous solution to form micelles. The commonly used hydrophilic monomer is polyethylene glycol (PEG), and the widely used core-forming blocks are poly(propylene oxide), poly(caprolactone), poly(D, L-lactic acid) and poly(L-aspartic acid). An A-B di-block structure ('A' being the hydrophilic polymer shell and B being the hydrophobic polymer core) or an A-B-A multi-block structure of co-polymers of different hydrophobicity or a graft co-polymer (hydrophilic backbone chain of a polymer grafted with hydrophobic blocks) can be used. Polymeric micelles offer many advantages concerning thermodynamic stability in physiological solution leading to their slow dissolution *in vivo*. Polymeric micelles have several advantages as drug carriers being inexpensive, safe and stable (Gaucher et al., 2010; Lu et al., 2013; Yokoyama 2014; Cabral & Kataoka, 2014).

Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles refer to nanoscale size particles prepared using lipids that remain solid at room temperature (or/and body temperature) (Figure 6). The lipid component may comprise a broad range of lipid and lipid-like molecules such as triacylglycerols or waxes. The commonly used lipids include fatty acids (stearic acid), triglycerides (e.g., tristearine), waxes (e.g., cetyl palmitate), or a mixture of the above lipids.

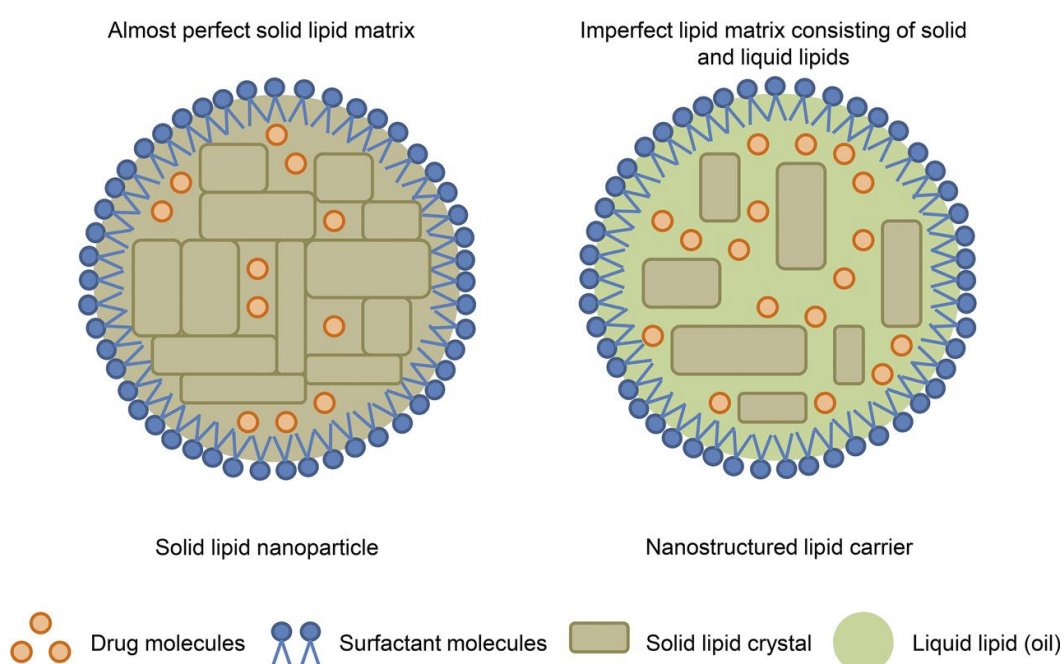


Figure 6. Morphology of solid lipid nanoparticle and nanostructured lipid carrier.
(From: *Lipid-Based Drug Delivery Systems in Cancer Therapy: What Is Available and What Is Yet to Come* (2016). DOI: 10.1124/pr.115.012070)

Depending on the type and concentration of the lipid, 0.5 to 5% emulsifier (surfactant) is added for the physical stabilization of the system, usually bile salts, phospholipids, sorbitan esters, ethoxylated fatty acids, or a mixture of these components. The diameter of such lipid particles can also be quite small, that is, in the range between 50 and 1000 nm dispersed either in water or an aqueous surfactant solution. The hydrophobic chains of surfactants are embedded in the fat matrix. Active ingredients can be solubilized homogeneously either in the core of the nanoparticles or the outside part.

The advantage of solid lipid nanoparticles as delivery systems for lipophilic active components lies in the immobilization of active elements by the solid particle structure leading to increased chemical protection, less leakage, and sustained release. This physical property allows better control of both the physical (against recrystallization) and chemical (against degradation) stability of the delivered constituents. Solid lipid nanoparticles are prepared by various techniques such as high-pressure homogenization, microemulsion formation, precipitation, and as lipid nanopellets and lipospheres. Although solid lipid nanoparticles possess several advantages, e.g., the use of physiological lipids, the avoidance of organic solvents in the preparation process, protection of sensitive molecules from the environment and controlled release characteristics, some disadvantages have been associated such as particle growth, unpredictable gelation tendency, polymorphic transitions and inherently low incorporation capacities due to the crystalline structure of the solid lipid. Besides, they are characterized by high biocompatibility, avoidance of organic solvent, excellent reproducibility even using different preparation methods. Other advantages include not only the ability to functionalize the solid lipid nanoparticles surface with markers and targeting devices in order to enhance the targeting process but also their ability to produce a sustained and slow release of the drug in the targeted site. The main limitations of solid lipid nanoparticles are a low loading capacity and leakage during storage (Mehnert & Mader, 2001; Parhi & Suresh, 2012; Ali & Singh, 2016; Lin et al., 2017).

Nanostructured lipid carriers, the new improved generation of lipid nanoparticles, have higher loading capacity and some less water in the dispersion due to the more complex lipid mixtures, both solid (fats) and liquid (oils) lipids at ambient temperature employed in the hydrophobic core (Figure 6). For example, a mixture of mono-, di-, or triglycerides with fatty acids of different chain length forms less perfect crystals, which can accommodate more hydrophobic compounds to avoid expulsion because of a less ordered inner structure. Like nano- and micro-emulsions, the major ingredients of nanostructured lipid carriers are lipid, surface active agent and water. In this case, a major portion of the oil constituent of the O/W emulsion is replaced by a solid lipid leading to a solid particle matrix of this carrier system at body temperature. To obtain the blends for the particles matrix, solid lipids are mixed with liquid lipids (oils), preferably in a ratio of 70:30 up to a ratio of 99.9:0.1. Solid lipid nanoparticles are composed of 0.1% to 30% (w/w) solid lipid dispersed in an aqueous medium and if necessary stabilized with preferably 0.5% (w/w) to 5% (w/w) surfactant while the overall solid content of nanostructured lipid carriers can be up to 95% (w/w). Less nanostructured lipid carriers are required to deliver similar drug content in comparison to solid lipid nanoparticles. Moreover, to produce nanoparticles powder, water removal from nanostructured lipid carriers is more cost-effective. Nanostructured lipid carriers are fabricated using various methods. The most used are the hot homogenization method, the cold homogenization method and solvent emulsification-evaporation method.

In the hot homogenization method, the drug is initially dissolved or dispersed in the melted lipid mixture. Then, the lipid melt is dispersed in aqueous emulsifier solution at the same temperature by high speed stirring/ shearing. The obtained hot emulsion may further be homogenized at the same temperature, by instruments such as high-pressure homogenizer, high intensity ultrasonic probe/jet/bath or microfluidizer, to produce a hot nanoemulsion. Subsequently, nanostructured lipid carriers are produced by cooling the hot nanoemulsion in cold water, room temperature or by a heat exchanger to crystallize lipid droplet and precipitate the lipid nanoparticles. In the cold homogenization method, after dissolving/dispersing bioactive compound in the melt lipid mixture, the bulk lipid is rapidly cooled. Subsequently, the bulk lipid matrix is milled to form lipid microparticles. It is important to make sure that during the milling process the temperature does not exceed the temperature of lipid with the lowest melting point. The microparticles are then dispersed in a cold emulsifier solution and further homogenized to produce fine lipid nanoparticles. In the solvent emulsification-evaporation method, lipids and bioactive compound are dissolved in a water-immiscible organic solvent with low boiling point. The solution is then emulsified in the aqueous emulsifier solution. Upon evaporation of the solvent under reduced pressure, the organic solvent will be removed leaving the lipids and bioactive compound, nanoparticles form. The other preparation methods are the solvent injection, the phase inversion technique, the multiple emulsion method and the membrane contractor techniques (Muller et al., 2002; Bhise et al., 2017; Khosa et al., 2018).

Microemulsions and nanoemulsions

Recently, there has been considerable interest for the micro and nanoemulsion formulation, for the delivery of hydrophilic as well as lipophilic drug as drug carriers and because they improve the drug's solubility and bioavailability, for the long shelf life and the ease methods of preparation. In 1959, Schulman and co-workers visualized the existence of small emulsion-like structures by electron microscopy and subsequently coined the term "microemulsions" (Figure 7). They are currently defined as homogeneous thermodynamically stable transparent dispersions of two immiscible liquids stabilized by an interfacial film of surfactants. Structurally, there are three different type of microemulsions: oil-in-water (o/w), water in oil (w/o) and bicontinuous. They have droplet size above 100-500 nm and require very low energy to formulate emulsion, since they form spontaneously when aqueous, oil and amphiphilic components are brought into contact, besides having a lower production cost compared to nanoemulsions (Talegaonkar et al., 2008; He et al., 2010). Nanoemulsions are non-equilibrium systems with a spontaneous tendency to separate into the constituent phases, prepared using lower surfactant concentrations than microemulsions (Figure 7).

Nevertheless, nanoemulsions may possess a relatively high kinetic stability even for several years, due to their very small size, essentially the consequence of significant steric stabilization between droplets. They have droplets covering the size range of 20–500 nm and referred to as mini-emulsions, ultrafine emulsions, and submicrometric emulsions. There is an application of high shear generally obtained by microfluid, or ultrasonic approach generally used to reduce the droplet size to nanoscale (Odriozola-Serrano et al., 2014; Ali et al., 2017).

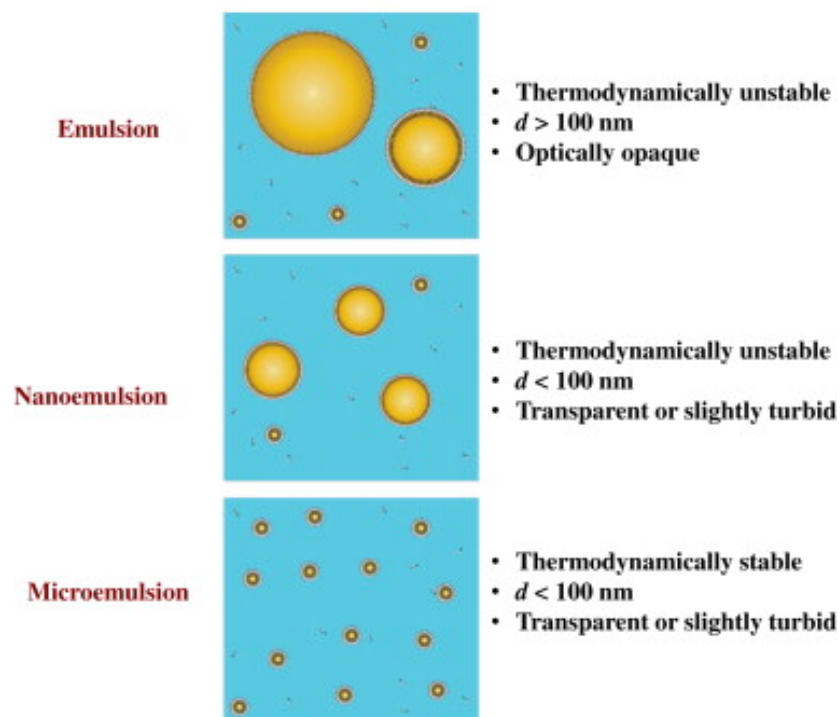


Figure 7. Schematic representation of different kinds of colloidal dispersions that can be used in the beverage industry: emulsions, nanoemulsions, and microemulsions.

(From: *Beverage emulsions: Recent developments in formulation, production, and applications* (2014).

DOI: 10.1016/j.foodhyd.2013.07.009)

A further formulation is represented by the self-microemulsifying drug delivery system, stable mixture of drug, oil, surfactant, and co-surfactant, which can form fine oil-in-water droplets with a diameter size of less than 100 nm under mild agitation of the gastrointestinal tract without the dissolution process. Self-microemulsifying drug delivery systems have the potential to deliver poorly water-soluble drugs because the microemulsion droplets provide 100% of the capacity to entrap a drug and protect the drug from gastrointestinal degradation. Moreover, the droplets can be rapidly dispersed in blood as well as lymph, and the lymphatic drug transport can avoid the first-pass effect. Additionally, the self-microemulsifying drug delivery systems are suitable for filling in gelatin capsules as the unit dosage container and stored at room temperature (Rahman et al., 2012; Dokania & Joshi, 2014).

Liposomes

The name liposome derives from the two Greek words “lipo” meaning fat and “soma” meaning body, which perfectly describes these spherical objects that are made mainly from natural or synthetic phospholipids (mainly phosphatidylcholine) and cholesterol that acts as a fluidity buffer (Figure 8).

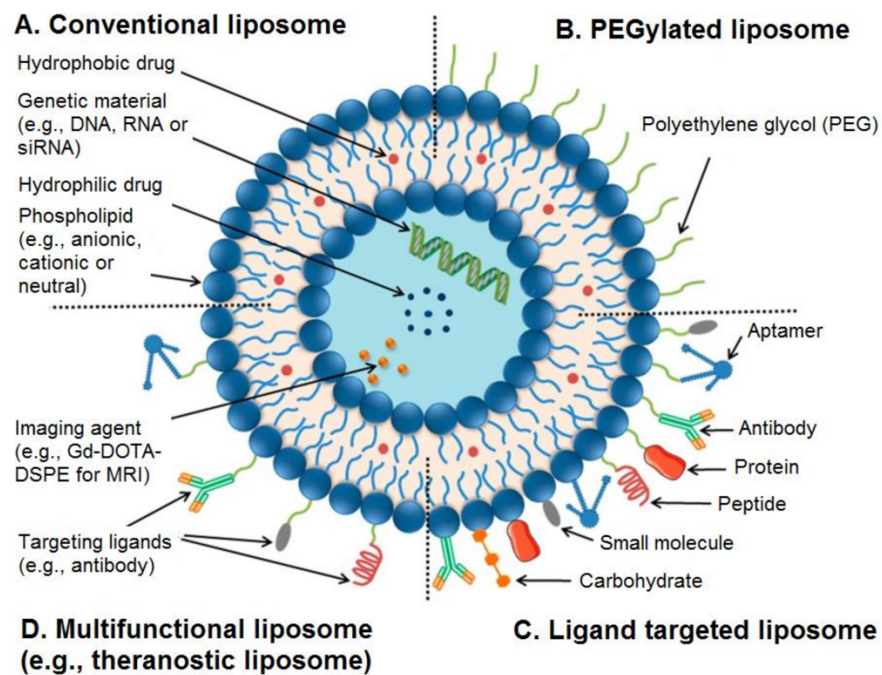


Figure 8. Liposomes: Conventional liposomes are made of phospholipids (A); PEGylated/stealth liposomes contain a layer of polyethylene glycol (PEG) at the surface of liposomes (B); targeted liposomes contain a specific targeting ligand to target a cancer site (C); and multifunctional such as theranostic liposomes, which can be used for diagnosis and treatment of solid tumors (D).

- (From: *Surface Functionalization and Targeting Strategies of Liposomes in Solid Tumor Therapy: A Review* (2018). DOI: 10.3390/ijms19010195)

Liposomes are one of the most studied colloidal delivery systems; in fact, they were first developed for drug delivery purposes as early as the 1970s.

Liposomes can contain one bilayer forming unilamellar vesicles (ULV), several concentric bilayers forming multilamellar vesicles or nonconcentric bilayers forming multivesicular vesicles (MVV). There are different types of liposomes, including Small Unilamellar Vesicles (SUV), Multilamellar Vesicles (MLV), Large Unilamellar Vesicles (LUV), Multivesicle Vesicles (MVV) and cochleate vesicles. The size of these structures can be rather small (in the range of 20 nm) or rather large (exceeding 1 μm). They exhibit many advantages concerning amphiphilic character, biocompatibility, and ease of surface modification rendering them suitable candidate delivery systems for biotech drugs. Both lipophilic and hydrophilic natural products could be loaded in liposomes in order to optimize their physical and chemical properties (Gregoriadis 1976; Lai et al., 2013; Sherry et al., 2013; Mallick & Choi, 2014; Bozzuto & Molinari, 2015).

Niosomes

Niosomes are non-ionic surfactant vesicles, formed when non-ionic surfactants (mainly of alkyl or dialkyl polyglycerol ether class) are added to cholesterol and a small amount of anionic surfactant such as diacyl phosphate with subsequent hydration in aqueous media. Addition of cholesterol provides rigidity to the bilayer leading to the formation of less permeable niosomes. The non-ionic surfactants increase the size of vesicles and provide charge to the vesicles and hence increase the entrapment efficiency of niosomes. These vesicular systems can be used as carriers of amphiphilic and lipophilic molecules. Niosomes are expected to be better drug carrier system than liposomes upon consideration of factors like cost, stability, entrapment efficiency and bioavailability (Rajera et al., 2011; Moghassemi et al., 2014).

Nanocochleates

Utilization of vesicles has limitations, principally due to poor mechanical stability due to leakage and fusion of the formulation and the limited utility in the oral administration. In this perspective, cochleates are the systems, which can overcome the limitations of liposome and satisfy the present needs of the market. Cochleates were first observed by Papahadjopoulos et al., 1975 using phosphatidylserine liposomes in the presence of Ca^{2+} or Mg^{2+} . Their simple structure is formed of negatively charged phospholipid bilayers through the interaction with multivalent counter ions as bridging agents between the bilayers (Figure 9).

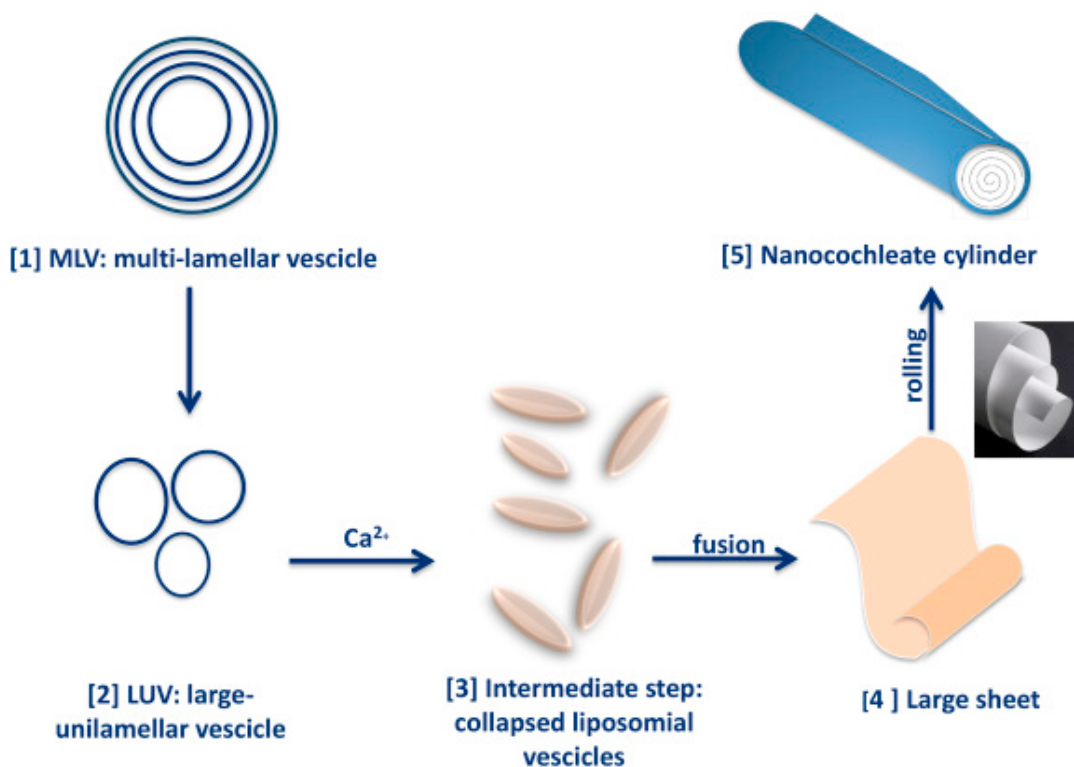


Figure 9. Schematic representation multi-step preparation process of nanocochleates.
(From: *Thyme essential oil loaded in nanocochleates: Encapsulation efficiency, in vitro release study and antioxidant activity* (2017).

DOI: 10.1016/j.lwt.2016.12.006)

Negatively charged phospholipids can serve as the main building blocks of cochleates. They present an elongated shape and a carpet roll like morphology invariably accompanied by tightly packed bilayers (cigar-like) having nano- and microstructures that consist of a series of phospholipid bilayers rolled up in a spiral or stacked sheets, with little or no internal aqueous space (Figure 9). Apart from being biocompatible, cochleates exhibit high stability, superior mechanical stability and better protection for encapsulated drugs compared with liposomes which are attributed to their unique compact structure. They can be stored by freeze-drying, maintaining their structure even after lyophilization and are easy to produce and they are considered suitable carrier of small hydrophobic molecules aiming to obtain smaller nanoparticles of less than 500 nm principally for oral delivery. Hence, unique features of the nanocochleates are water-free interior, rod-shape & rigid stable structure making them a great platform for delivery of small hydrophobic drugs with a poor bioavailability (Zarif & Mannino, 2000; Zhong et al., 2018).

Cyclodextrins

Cyclodextrins are natural macrocyclic oligosaccharides well known for having toroid-shaped structures with rigid lipophilic cavities and a hydrophilic outer surface ensuring good dissolution of the complex in an aqueous environment. They are able to entrap a variety of organic compounds in their cavities, which can influence the dissolution rate, the aqueous solubility of poorly water-soluble substances and their stability in the presence of light, heat and oxidizing conditions, as well as modify physico-chemical properties of drugs. The major advantages of the use of cyclodextrin-complexation are protection of the active ingredients against oxidation, light-induced reactions, decomposition and thermal decomposition, loss by evaporation and sublimation, and elimination or reduction of undesired tastes/odours. They also can reduce or prevent gastric-intestinal irritation (mainly due to anti-inflammatory drugs) or ocular disturbances, prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders and to reduce microbiological contamination, fibers, or the elimination of other undesired components and hygroscopicity. Moreover, the formation of inclusion complex increases the guest's *in vivo* stability against hydrolysis, oxidation, decomposition, and dehydration, consequently increasing bioavailability and bioefficacy.

There are three main types of cyclodextrins: α -, β -, and γ -cyclodextrins corresponding to 6, 7, and 8 glucopyranose units linked by α -(1,4) bonds, respectively. The dimensions of the internal cavity are 0.5–0.8 nm and are crucial for the “encapsulation” of guest molecules (Figure 10). An increasing number of CDs derivatives is also available, randomly methylated-CDs, hydroxypropyl-CDs, and low methylated-CDs (Kurkov & Loftsson, 2013; Crini, 2014; Pinho et al., 2014).

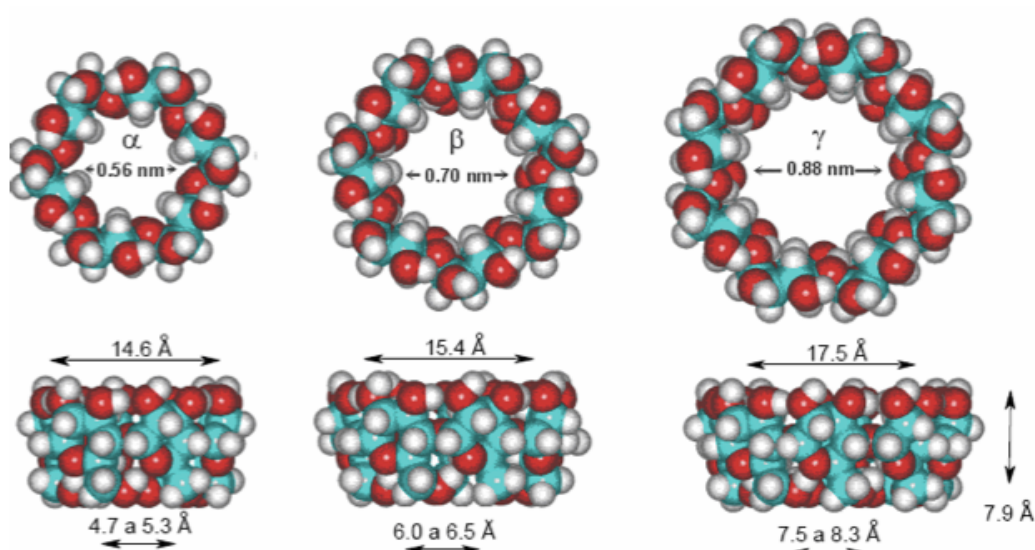


Figure 10. Schematic representation of different kinds of cyclodextrins.
(From: <https://scientiablog.com/2013/11/28/la-molecula-de-la-triple-accion/>)

Aim of the work

In this research, the utility of nano-drug delivery systems to improve the biopharmaceutical features and the pharmacological potential of plant-derived molecules was highlighted. In particular, lipid-based nanocarriers, such as microemulsions/nanoemulsions (in this work these terms were used indistinctly, referring only to the nanoscopic dimensions of the dispersed droplets), solid lipid nanoparticles and chitosan-coated solid lipid nanoparticles, nanostructured lipid carriers and liposomes were developed for the oral delivery and brain delivery of silybin, the major biologically active component in silymarin, *Vitex agnus-castus* L. extract, silymarin, the extract derived from seeds and fruits of *Silybum marianum* L. Gaertn., Salicis cortex extract and andrographolide, the major diterpenoid in the traditional Asian medicinal plant *Andrographis paniculata* (Burm. f.) Wall. ex Nees. Furthermore, the influence of chitosan-coating on human serum albumin nanoparticles was studied to propose a novel drug delivery system for the nose-to-brain delivery of natural active compounds. More detailed and specific information about the state of art of the formulations and plant-derived compounds are reported in the introduction section of each chapter of this thesis.

**NANOEMULSION FOR IMPROVING SOLUBILITY AND PERMEABILITY
OF VITEX AGNUS-CASTUS EXTRACT: FORMULATION AND IN VITRO
EVALUATION USING PAMPA AND CACO-2 APPROACHES**

INTRODUCTION

In the last years, the use of herbal medicines has been increased due to their therapeutic effects and fewer adverse effects as compared to the modern medicines. However, many herbal drugs and herbal extracts have low *in-vivo* activity due to their poor solubility, absorption and hence poor bioavailability, low stability or high metabolism.

Drug delivery systems represent useful tools to formulate herbal drugs and to enhance their bioavailability. The novel formulations have remarkable advantages over conventional formulations of natural compounds and extracts, which include increase of solubility, bioavailability, stability, enhancement of intracellular uptake, modification of pharmacokinetics and biodistribution, sustained delivery. The strategy of drug delivery systems has been applied not only to many synthetic drugs but also to phytopharmaceuticals, including both isolated compounds and purified extracts (Porter et al., 2008; Sermkaew et al., 2013; Bergonzi et al., 2013, 2014; Righeschi et al., 2014; Esfanjani-Manesh et al., 2016; Isacchi et al., 2017).

The purpose of this study was to develop an innovative oral formulation of *Vitex agnus-castus* extract (VAC extract) to improve the solubility of constituents and enhance its therapeutic efficacy.

In recent years, much attention has been focused on lipid-based nanovectors, namely solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), self-emulsifying drug delivery systems (SEDDSs) and nanoemulsions (NEs) (Ibrahim et al., 2015). Lipid carriers promote absorption through the GI tract, by ameliorating the solubility of the drug and by increasing the solubilization capacity of the GI fluids (Porter et al., 2007, 2008). In fact, lipid formulations avoid the dissolution step in comparison with an oral solid or suspension dosage form and provide significant improvement of oral absorption.

NEs are highly dispersed, stable, transparent formulations and easy to prepare. Furthermore, their nanoscopic dimensions allow a better absorption by the cell membranes. Finally, NEs showed the ability to solve the problems of solubility and stability of many synthetic drugs, phytotherapeutic, nutraceuticals and food additive (Čilek et al., 2006; Hu et al., 2011; Sermkaew et al., 2013; Bergonzi et al., 2014; Akhtar et al., 2016). These characteristics give them transparency and allow to formulate the drug as both aqueous solutions and as non-aqueous concentrates, diluted with water immediately before administration, or administered as such.

The main aim of this research was to formulate an o/w nanoemulsion of VAC for oral administration, using acceptable food components, to increase solubility, stability and ameliorate intestinal permeability of extract's constituents.

VAC is a native species of the Mediterranean region. It has a long tradition as an herbal remedy and it was used in ancient times not only as an aphrodisiac but also against several disturbances of the female genital system. Different classes of analytes, like essential oil, iridoids, flavonoids, phenolic acids and diterpenoids, have been reported from VAC fruits and preparations thereof (Hoberg et al., 1999; Prestos et al., 2006; Högnér et al., 2013).

Therapeutic indications are premenstrual syndrome including symptoms such as mastodynia or mastalgia and menstrual cycle disorders (Berger et al., 2000). *In vitro* and *in vivo* experiments have demonstrated the dopaminergic and prolactin-inhibiting activities of VAC extract. Indeed, an increase in serum levels of prolactin is a frequent cause of menstrual irregularities, pre-menstrual syndrome and mastodynia (Jarry et al., 1994; Zahid et al., 2016).

The main characteristic constituents are: bicyclic diterpenes, iridoid glycosides (agnuside), lipophilic flavonoids (casticin, penduletin), hydrophilic flavones (luteolin, isovitexin) (Zahid et al., 2016).

The study also includes the optimization of HPLC-DAD-ESI/MS analytical method for qualitative and quantitative characterization of commercial VAC extract and the preparation of nanoemulsion as lipid carrier of phytocomplex. Physical and chemical stabilities of the optimized formulation were assessed for three months. Finally, *in vitro* permeation or transport studies were performed by using different models such as parallel artificial membrane permeability assay (PAMPA) and Caco-2 cells line, after preliminary cytotoxicity studies. These *in vitro* results could provide useful information about dissolution and permeation/absorption aspects, which further could be correlated to *in vivo* behaviour. PAMPA was performed in order to estimate passive transcellular permeability. It is a non-cell based permeability model but is considered robust, reproducible and fast. This *in vitro* assay resulted useful not only in the screening of single molecules, but also for complex matrices, such as extracts and their formulations.

Considering that most compounds reach the blood stream by passive mechanism, PAMPA has been proposed as helpful complement to Caco-2 assay to predict the oral absorption of VAC extract. Caco-2 cells represent the most widely and successfully model used to predict passive diffusion, active transport, paracellular permeability and active efflux.

MATERIALS

VAC dry extract BNO 1095 (DER 7-11:1), was supplied by Bionorica SE, Neumarkt, Germany. Agnuside, isovitexin and casticin HPLC grade, luteolin, apigenin, isoorientin, ac. 3,4-dihydroxybenzoic and ac. p-OH-benzoic were from Extrasynthese (Genay Cedex, France).

Olive oil was from Olivia (Badia a Settimo, Florence, Italy); wheat germ oil (*Triticum aestivum*) was purchased from Aboca (Sansepolcro, Arezzo, Italy); sunflower oil (*Helianthus Annus*) was from Coop (Massarosa, Lucca, Italy); Tween 20 (Polyoxyethylene (20) sorbitan monolaurate), Tween 40 (Polyoxyethylene sorbitan monopalmitate), Cremophor EL (Castor Oil Fatty Acids, Ethoxylated Glycerol Esters), DL- α -Tocopherol acetate (Vitamin E), Triacetin, PEG 400, were purchased from Sigma-Aldrich (Milan, Italy); hempseed oil, almond oil, borage oil, glycerol, propylene glycol were from Galeno SrL (Comeana, Prato, Italy); oleic acid was from Farmitalia, Carlo Erba SpA (Milan, Italy); Labrafil (2-[2,3-bis(2-hydroxyethoxy)propoxy]ethanol; hexadecanoic acid; octadecanoic acid), Labrasol ECH (Caprylocaproyl polyoxyl-8 glycerides), Capryol 90 (Propylene glycol monocaprylate), Transcutol HP (Diethylene glycol monoethyl ether) were from Gattefossé (Saint Priest, France).

Ethanol analytical reagent, Acetonitrile and Methanol HPLC grade and formic acid ($\geq 98\%$) were purchased from Sigma-Aldrich (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, USA). Cholesterol, Lecithin, Dichloromethane, DMSO, 1,7-Octadiene ($\geq 98\%$), Phosphate buffered saline (PBS) bioperformance certified, lipase from porcine pancreas, pepsin from porcine gastric mucosa, bile salts, HCl were purchased from Sigma-Aldrich (Milan, Italy).

METHODS

Preparation of sample solution of VAC extract

Commercial extract (500 mg accurately weight) was suspended in 20 mL of methanol, ultrasonicated for 30 min and filtered. The extraction was repeated three times. The filtrate was evaporated until dryness, and the residue was dissolved in methanol and analyzed by HPLC-DAD-MS analysis. Aqueous solubility was determined by dissolving until saturation commercial extract in deionized water at room temperature. The residue was eliminated by centrifugation and the solution was analyzed by chromatographic analysis.

HPLC-DAD-MS analyses

The quantitative analyses were carried out using a HP 1100 liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Santa Clara, CA, USA). A 150 mm x 4.6 mm i.d., 5 μ m Zorbax Eclipse XDB, RP18 column was used (Agilent Technologies, Santa Clara, CA, USA). Chromatography was carried out in gradient mode using a flow rate of 0.6 mL/min. The mobile phase was (A) formic acid/water pH 3.2 and (B) CH₃CN. The multi-step linear solvent gradient used was: 0-5 min 15-20% B; 5-7 min 20-30% B; 7-10 min 30-40% B; 10-15 min 40-50% B; 15-20 min 50-80% B; 20-25 min 80-15% B; post time 10 min; oven temperature 30°C, flux: 0.6 ml/min. The UV/Vis spectra were recorded in the range 200-700 nm and the chromatograms were acquired at 210, 260, 280, 350 nm.

For qualitative analysis, MS experiments were conducted using a LTQ equipped with an ESI interface (Finnigan LTQ, Thermofisher Scientific, Waltham, Massachusetts, USA). Mass spectrometry and electrospray operating parameters were optimized for negative polarity. The following final settings were used: Sheath Gas Flow Rate (arb): 30, Aux Gas Flow Rate (arb): 5, Sweep Gas Rate (arb): 5, Capillary Temp (°C): 290.00, Capillary Voltage (V): 16.93, Tube Lents (V): -99.72.

Method validation

HPLC-DAD method was validated according to current international regulatory guidelines (ICH, 1996; EMA, 2012). In particular, linearity, accuracy, precision, reproducibility, repeatability of the method, limit of quantification (LOQ) (Beloqui et al., 2016) and limit of detection (LOD) were assessed.

Calibration standard series of casticin, isovitexin and agnuside were analyzed in triplicate. Flavonoids of extract were expressed as isovitexin and casticin, agnuside was used to quantify iridoids. Calibration curves were obtained by plotting the peak areas versus the concentrations of each standard compound. The regression parameters (intercept, slope and correlation coefficient) were calculated by linear regression analysis.

The reproducibility of the injection integration procedure was determined for agnuside (1.96 mg/mL), casticin (1.97 mg/mL) and isovitexin (1.92 mg/mL). The solutions were injected ten times and the relative standard deviation (R.S.D.%) values were calculated.

Three solutions at different concentrations (2.08, 3.17 and 4.26 mg/mL of extract in methanol) were prepared in order to evaluate the repeatability of the method. Each solution was injected three times. The contents of iridoids and flavonoids derivatives were calculated in order to estimate the R.S.D.%. The same samples used to determinate the repeatability of the method were injected six times on three different days for the determination of intermediate precision.

Accuracy was assessed by analyzing the recovery percentage of standards into the three preparations of VAC extract. Three independent solutions of extract were prepared (2.00, 2.67 and 4.00 mg/mL) and each sample was injected three times.

The average recovery percentage was calculated for each level of concentration. The accuracy was determined by spiking 100 µg/ml of casticin, isovitexin and agnuside separately to the three batches of the extract.

Specificity was defined as the non-interference by other analytes detected in the region of interest.

The peak purity was investigated by inspecting the UV-spectra and MS spectra at the beginning, at the apex and at the end of the peaks of each constituent of the extract. No deviations were reported.

The LOQ (Beloqui et al., 2016), defined as the lowest concentration of analyte that could be quantified with acceptable accuracy and precision, was estimated by injecting a series of increasingly dilute standard solutions until the signal-to-noise ratio was reduced to 10. The LOD corresponds to the concentration of the analyte, which provides a signal equal to the background (blank) plus three times the standard deviation of the blank.

Solubility studies

The solubility of VAC extract in water, oils (olive oil, triacetin, wheat germ oil, sunflower oil, tocopherol acetate, hempseed oil, almond oil, borage oil, oleic acid), surfactants and cosurfactants (Tween 20, Tween 40, cremophor EL, Labrafil, labrasol ECH, PEG 400, ethanol, glycerol, propylene glycol, Capryol 90, Transcutol HP) was determined by suspended an excess of extract in 5 mL of each vehicles. Each mixture was shaken at 25°C for 24 h, and then was centrifugated at 13148 x g for 10 min. After the removal of the supernatant, the concentration of the components of the extract was determined by HPLC at 260 nm after dilution with methanol/dichloromethane (3:2 v/v).

Construction of ternary phase diagram

Pseudoternary phase diagrams were constructed, in order to obtain the range of concentration in which NE exist. The pseudoternary phase diagrams were constructed using the water titration method.

Surfactant and cosurfactant were mixed at different weight ratios (S_{mix}). For each S_{mix} ratio, pseudoternary phase diagram was elaborated by testing weight ratio of oil- S_{mix} of 0:100, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. Each oil- S_{mix} mixture was diluted under vigorous stirring dropwise with water. After equilibrium, each sample was visually checked and determined as being clear nanoemulsion, emulsion or gel.

Extract-loaded NE was prepared by dissolving powder into the selected oil- S_{mix} mixture, adding the required quantity of water, and stirring to form a clear and transparent dispersion. The resulting NE was tightly sealed and stored at 4°C temperature.

Solubility of VAC extract in NE

The NE capacity to solubilize VAC extract was investigated and compared with the solubility of extract in aqueous solution. To determine the maximum loading capacity of NE, increasing amounts of extract (ranging from 5 to 60 mg) were loaded to the optimized formulation. The mixture was stirred for 24 h at 25°C under light shielding. The undissolved drug was removed by centrifugation at 13148 x g for 10 min, then the supernatant was taken, and the constituents were quantified by HPLC-DAD at 260 nm after dilution with methanol/dichloromethane (3:2 v/v). The analyses were performed in triplicate.

Particle size analysis and ζ -potential

Droplet size of the developed NE was measured by a Dynamic Light Scattering (DLS), Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25°C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, Pdl) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the Cumulants method, fitting a single exponential to the correlation function to obtain particle size distribution. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle, diluting the samples in distilled water. ζ -potentials were measured using the same instrument; for all samples, an average of three measurements at stationary level was taken. The temperature was kept constant at 25°C by a Haake temperature controller. ζ -potential was calculated from the electrophoretic mobility, using the Henry correction to Smoluchowski's equation.

Morphological characterization

Morphology and structure of NE were studied using transmission electron microscope (TEM, Jeol Jem 1010, Tokyo, Japan). 5 μ L of NE was applied to a carbon film-covered copper grid after appropriate dilution. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen, which was stained with a phosphotungstic acid solution 1% w/v in sterile water. The samples were dried for 3 min and then were examined under electron microscope and photographed at an accelerating voltage of 64 kV.

Stability studies

Empty and extract-loaded (60 mg/mL) samples were inserted into sealed glass vials and stored at 4°C for 2 months, to evaluate the stability of NEs. Their chemical and physical stabilities were studied by monitoring the occurrence of phase separation, dispersed phase size and drug content at predetermined intervals by DLS and HPLC-DAD analyses.

Furthermore, to mimic physiological dilution process after oral administration, the NEs were diluted 10, 20 and 30-fold with distilled water (pH = 5.5). The dilutions were followed by gentle vortexing for 2 min at room temperature. The samples were analyzed by DLS to confirm the physical stability of the systems in terms of size and homogeneity. Chemical stability was assessed by quantifying total flavonoids and iridoids over 2 months.

NEs were also diluted up to 20-folds in media with different pH to mimic diverse environments met after oral administration.

The intragastric stability was tested in simulated gastric fluid (SGF) as described earlier (Aditya et al., 2013) . Briefly, 5 ml of NE were suspended in 5 mL SGF (0.32% w/v pepsin, 2 g of sodium chloride and 7mL HCl dissolved in 1 L water and pH adjusted to 1.8 using 1M HCl) and incubated in a water bath at 37 °C under shaking speed of 100 strokes/min. After 2 h, the sample was collected to analyze the size and Pdl.

After digestion in simulated stomach condition, previous sample was subjected to digestion under simulated intestinal condition containing intestinal enzyme complex (lipase 0.4 mg/mL, bile salts 0.7 mg/mL, and pancreatin 0.5 mg/mL) and calcium chloride solution 750 mM at pH 7.0, 37 °C under shaking speed of 100 strokes/min. After 2 h digestion in simulated intestinal fluid (SIF), sample was collected and its physical stability was checked by DLS analysis.

In vitro parallel artificial membrane permeability assay (PAMPA)

The assay was carried out in a 96-well, MultiScreen-IP PAMPA (Millipore Corporation, Tullagreen, Carrigtwohill, County Cork, Ireland) filter plate. The ability of compounds to diffuse from a donor compartment, through a PVDF membrane filter pre-treated with a lipid-containing organic solvent, into an acceptor compartment was evaluated. 5 μ L of lecithin (10g/L) and cholesterol (8g/L) in 1,7-octadiene solution were added to the filter of each well. Immediately after the application of the artificial membrane, 250 μ L of drug-containing donor solutions (saturated solution of extract in water and VAC loaded NE, 60 mg/mL) were added to each well of the donor plate. 250 μ L of buffer (0.05 mL/mL DMSO/PBS, pH 7.4) were added to each well of the acceptor plate. Then, the acceptor plate was placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. The plate was covered and incubated at room temperature and permeation was evaluated at 0.5, 2, 4 hours.

Cell Culture

The human colon carcinoma cell line Caco-2 was kindly provided by Prof. Masini (University of Florence, Italy). Cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. Caco-2 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Upon reaching 80% confluence, cells were sub-cultured weekly at a split ratio of 1:3 by trypsinization.

MTS assay for cell viability

Viability analysis was performed using Cell Titer 96™ Aqueous One solution cell proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay kit (Promega Madison, WI, USA). In brief, Caco-2 cells were transferred to flat bottom 96-well tissue culture plates (Corning, USA) at a seeding density of 5×10^3 cells/well and allowed to grow for 24 h under the conditions detailed above. For MTS assay, the culture medium was removed and replaced with fresh medium containing VAC loaded NE (60 mg/mL; 1:100-1:800 dilution) and the cells were incubated for a further 4 or 24 h. Then the cells were exposed to MTS solution and allowed to incubate for 2 h at 37°C. The product of the reaction was measured at 490 nm using a spectrophotometer (Multilabel Counter 1240 Victor 3, Perkin Elmer, Waltham, MA, USA). Cell death was expressed as a percentage of values obtained from control, untreated cells calculated from three replicates of each NE dilution.

Cell culture for Transport Studies

For transport studies, cells were seeded at 50000 cells/well in cell culture inserts with PET (polyethylene terephthalate) membranes (0.4 μm pore size, 1.12 cm^2 growth surface area; BRAND, Italy). Culture medium (DMEM) was added to apical (AP) (0.5 mL) and basolateral (BL) (1.5 mL) side, and was replaced every other day for the first week and daily after that. Cells were let to differentiate for 18-21 days.

Monolayer integrity

The integrity of the layer was evaluated with the lucifer yellow (LY) permeability assay according to Iacomino et al., 2013. LY was diluted in transport buffer (HBSS with Ca^{2+} , Mg^{2+} , 25 mM HEPES, pH 7.4) and added to the AP compartment at a final concentration of 100 μM . After incubation at 37 °C for 1 h, the HBSS in the BL chamber was collected, and the concentration of LY was determined by using 485 nm excitation and 530 nm emission on a fluorescence plate reader (Multilabel Counter 1240 Victor 3, Perkin Elmer, Waltham, MA, USA). The percentage of AP to BL permeability was calculated according to the following equation:

$$\% \text{ permeability} = \frac{(\text{Fluorescence in the BL} - \text{blank})}{(\text{Fluorescence LY} - \text{blank})} * 100$$

The maximum critical flux of LY to identify leaky monolayers was estimated to be less than 3% of starting concentration.

Transport experiments

Transport study was performed according to Hubatsch et al., 2007. Before the transport study, the culture medium (DMEM) was replaced with preheated (37°C) transport HBSS medium supplemented with 25 mM HEPES (pH 7.4). After the cell monolayer was equilibrated for 30 min at 37°C, Caco-2 cells were treated for 1 h with different dilutions of VAC-loaded NE (1:5; 1:10; 1:15) in HBSS in the AP chamber, while the BL chamber contained only HBSS.

At predetermined time intervals (0 to 60 min) 0.3 mL of medium in the BL side was taken for HPLC analyses and replaced with the same volume of fresh HBSS. At the end of the experiments, the integrity of the layer was re-evaluated with the LY permeability assay as described above (Iacomino et al., 2013).

Statistical analysis

Experiments were repeated three times and results expressed as a mean $\pm$ standard deviation. Statistical significance was calculated by Student's t-test with statistical significance level set at $P < 0.05$.

RESULTS AND DISCUSSION

HPLC-DAD-ESI/MS analytical methods

The first step of the investigation was the HPLC-DAD-ESI/MS characterization of VAC extract. The wavelengths of 210, 260, 280 and 350 nm were selected for acquiring chromatograms of iridoid glycosides (agnuside), benzoic acid derivatives, flavonoids (isoorientin, isovitexin, luteolin, apigenin, penduletin, casticin), diterpenoids. Various linear gradients of acetonitrile–water and methanol–water were investigated at different flow-rates, by using various columns and temperature conditions, in order to achieve better chromatographic separation. Finally, the gradient programme described in the experimental part was selected, because all the peaks resulted clearly separated. Figure 2 reports the HPLC-DAD profile of the methanolic extract of at the wavelength of 260 nm. The identification of compounds was obtained by comparing UV spectrum, retention time and MS data with those of standards or with the data reported in the literature.

The HPLC-DAD method was validated according to the criteria established by ICH guidelines, 1996, relatively to the following compounds: agnuside, isovitexin and casticin (Figure 1).

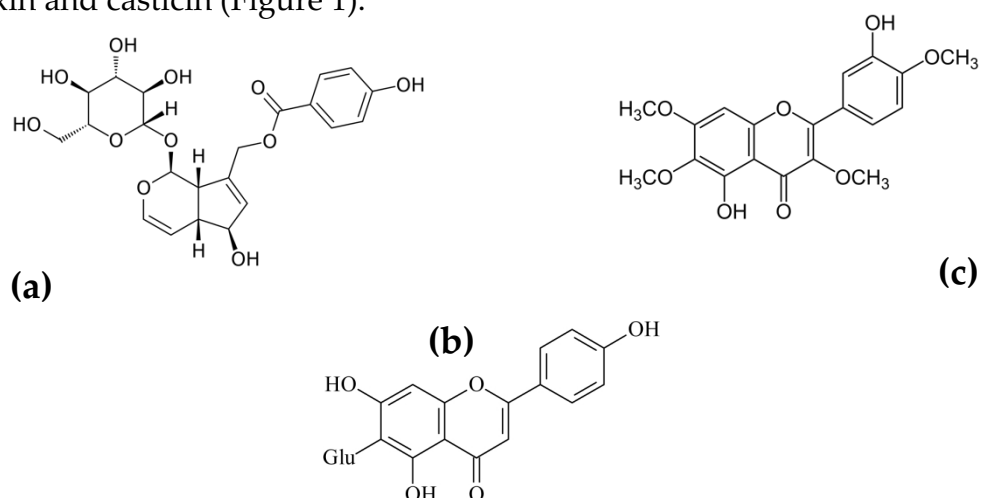


Figure 1. Chemical structure of agnuside (a), isovitexin (b) and casticin (c).

Linearity

Linearity range of response was determined for agnuside, isovitexin and casticin. They showed a linear response from 1 to 1000 µg/ml and all the curves had coefficients of linear correlation $R^2 \geq 0.999$.

Additionally, the slope R.S.D. % values were lower than 1.5%, in particular for the casticin was 0.69%, for the agnuside was 0.24% and for isovitexin was 0.51 % which indicated a high accuracy of the method.

Reproducibility

The reproducibility of the injection integration procedure was determined for agnuside (1.96 mg/ml), casticin (1.97 mg/ml) and isovitexin (1.92 mg/ml). The solutions were injected ten times and the relative standard deviation (R.S.D. %) values were calculated.

R.S.D. % of agnuside was 0.37%, the value for casticin was 0.58% and for isovitexin was 0.66%.

Repeatability of the method

Agnuside (R.S.D. % 0.74%, 1.60% and 0.83% respectively), casticin (R.S.D. % 0.48%, 0.18 % and 0.64%) and isovitexin (R.S.D. % 1.92%, 1.76% and 1.04%).

Intermediate precision

Results of were reported in Table 1.

Accuracy

The recovery percentages of standards spiked into VAC extract at three different concentrations were calculated. Three independent solutions of extract were prepared (2, 2.67 and 4 mg/mL) and each sample was injected three times. The average percentage recovery was calculated for each level of concentration. The accuracy was determined by spiking 100 µg/mL of casticin and isovitexin separately to the three batches of the extract.

The percentages recovery of casticin standard spiked into three preparations were $103.07 \pm 0.43\%$; $94.01 \pm 0.67\%$ and $103.97 \pm 0.82\%$, respectively; for isovitexin were $105.72 \pm 0.36\%$; $114.70 \pm 1.19\%$ and $126.89 \pm 1.96\%$, respectively and for agnuside resulted $102.04 \pm 0.57\%$, $105.38 \pm 0.67\%$ and $113.62 \pm 0.52\%$, respectively.

Specificity

The peak purity was investigated by inspecting the UV-spectra and MS spectra at the beginning, at the apex and at the end of the peaks of each constituent of the extract. No deviations were seen.

Detection (LOD) and quantitation limit (LOQ).

LOD and LOQ of agnuside, casticin and isovitexin were determined by calculation of the signal-to-noise ratio. LOD resulted 0.98 ng for agnuside, 0.98 ng for casticin and 3.84 ng for isovitexin. LOQ resulted 1.96 ng for agnuside, 1.97 ng for casticin and 5.76 ng for isovitexin.

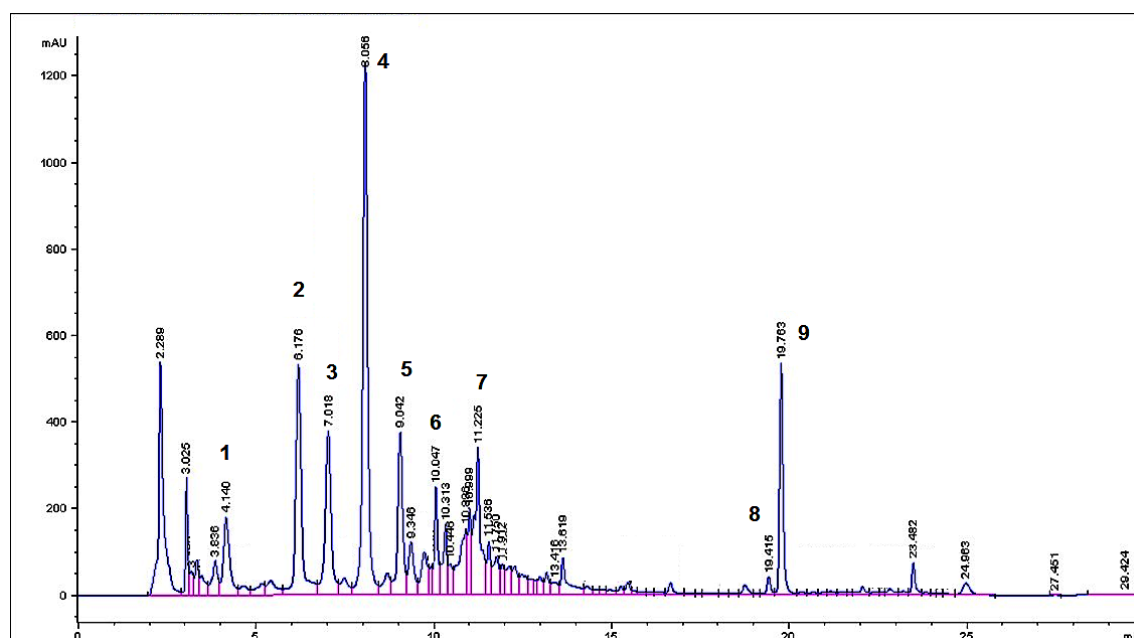


Figure 2. HPLC-DAD profile of VAC extract at 260 nm.

1. 3,4-Dihydroxybenzoic acid; 2. 4-Hydroxybenzoic acid; 3. Isoorientin; 4. Agnuside; 5. Isoviteixin; 6. Luteolin; 7. Apigenin; 8. Penduletin; and 9. Casticin.

Extract (mg/mL)	Day	RSD % Intraday			RSD % Interday		
		Agnuside	Casticin	Isovitexin	Agnuside	Casticin	Isovitexin
2.08	1	1.672	1.807	0.704			
	2	1.949	0.429	0.247	1.632	0.919	0.878
	3	1.276	0.523	1.685			
3.17	1	1.835	0.211	1.984			
	2	1.975	2.487	2.616	1.776	1.281	2.348
	3	1.519	1.203	2.444			
4.26	1	1.365	0.863	0.364			
	2	1.785	0.859	1.491	1.574	0.749	1.362
	3	1.574	0.525	2.231			

Table 1. Intermediate precision: RSD % values intraday e interday.

Solubility study

To find out appropriate constituents of NE, the solubility of extract was determined in various oils, surfactants and co-surfactants. The excipients chosen needed to be pharmaceutically acceptable. Higher solubility of the substances in the oil phase was another important criterion, as it would help the nanoemulsion to maintain the product in solubilized form.

Selected oils were olive oil, triacetin, wheat germ oil, sunflower oil, tocopherol acetate, Labrafil (oleoyl macrogol-6-glycerides), hempseed oil, almond oil, borage oil, oleic acid.

The surfactants were tween 20, tween 40, Cremophor EL, Labrasol ECH, PEG 400 and ethanol, glycerol, propylene glycol, Capryol 90, Transcutol HP were used as co-surfactant. The solubility was assessed by using the HPLC method previously reported.

The solubility's results were reported in Table 2 and expressed as total flavonoids and total iridoids.

The aqueous solubility of flavonoids resulted 0.313 ± 0.010 mg/mL (isoorientin 0.090 ± 0.011 mg/mL, casticin 0.031 ± 0.010 mg/mL, apigenin 0.077 ± 0.009 mg/mL, luteolin 0.079 ± 0.017 mg/mL, isovitexin 0.036 ± 0.014 mg/mL), and iridoids 0.172 ± 0.020 mg/mL (agnuside).

Oil solubility of the extract was low and only flavonoids can be quantified. Surfactants showed a higher solubilizing effect than oils in the case of both flavonoids and iridoids. This is probably because they are amphiphilic molecules with affinity for both polar groups and lipophilic groups of the molecules. The highest values were obtained with Cremophor EL, Transcutol HP and PEG 400. Selected media differently influences the solubility of the constituents, compared to water. In particular, olive oil, oleic acid, hemp oil, PEG 400, ethanol, propylene glycol and Transcutol increased the solubility of casticin; Transcutol, propylene glycol, PEG 400 and Tween 40 showed their positive effect on solubility of apigenin and isovitexin, while PEG 400 and propylene glycol ameliorated the solubility of isoorientin. PEG 400 and Chremophor increased solubility of agnuside. The solubility into single vehicles resulted lower than water, but a synergic effect of NE was observed for all types of constituents, both polar and lipophilic, as further reported.

The first NE tested by solubility results contained olive oil as lipophilic phase, ethanol, propylene glycol, Cremophor EL or Transcutol HP as co-surfactant, and Tween 20 as surfactant, but no clear and transparent systems were obtained, also at high surfactant concentrations (efficiency of the surfactant $> 90\%$ w/w and the water solubilization capacity < 1). This is because olive oil contains predominantly long-chain triglycerides of oleic acid and, in contrast to mineral oil, it is polar. However, molecular weight of both of oils is most probably too high to assist in the formation of a nanoemulsion.

Then, triacetin was tested as oil phase, and Labrasol and Cremophor EL as surfactant and co-surfactant, respectively. The ratio of oil to S_{mix} was changed during preformulation study: in particular, S_{mix} 1:1, 1:2, 2:1, 1:2.5, 1:3, 1:4 were considered. The optimized NE was assessed for clarity and transparency by visual inspection.

Oils	Flavonoids (mg/mL)	Iridoids (mg/mL)
Olive oil	0.107 ± 0.046	-
Triacetin	0.136 ± 0.036	-
Tocopheryl Acetate	0.008 ± 0.001	-
Labrafil	0.014 ± 0.003	0.01
Sunflower oil	0.058 ± 0.001	-
Hempseed oil	0.057 ± 0.010	-
Almond oil	0.022 ± 0.006	-
Wheat germ oil	0.066 ± 0.017	-
Borage oil	0.053 ± 0.013	-
Oleic acid	0.082 ± 0.001	-
Surfactant/Cosurfactant		
Tween 20	0.080 ± 0.016	0.065 ± 0.001
Tween 40	0.078 ± 0.016	0.023 ± 0.005
Cremophor EL	0.367 ± 0.008	0.303 ± 0.022
Labrasol	0.178 ± 0.012	0.130 ± 0.015
Capryol 90	0.057 ± 0.011	0.019 ± 0.002
PEG 400	0.214 ± 0.021	0.228 ± 0.041
Transcutol HP	0.206 ± 0.008	0.123 ± 0.037
Ethanol	0.163 ± 0.017	0.142 ± 0.017
Propylene glycol	0.170 ± 0.006	0.172 ± 0.062
Glycerol	0.043 ± 0.001	-

Table 2. Solubility of VAC constituents in different vehicles.

Mean $\pm$ SD; n=3.

Pseudoternary phase diagram study

Phase diagram was constructed in order to obtain the concentration range of components necessary for the existence of NE. The pseudoternary phase diagram was built using the water titration method. After equilibrium, each sample was visually checked to define if clear NE, emulsion or gel was present. Figure 3 shows the pseudoternary diagram with a field of the existence of NE. The selected composition (1:2 Oil- S_{mix} and S_{mix} 1:1) was: triacetin (20%) as the oil phase, labrasol (22%) as surfactant, Cremophor EL (22%) as cosurfactant (S_{mix}) respectively and water (36%). The NE contains 60 mg/ml of extract. Distilled water was used as an aqueous phase and it was added drop by drop, under gentle agitation, to each oily mixture.

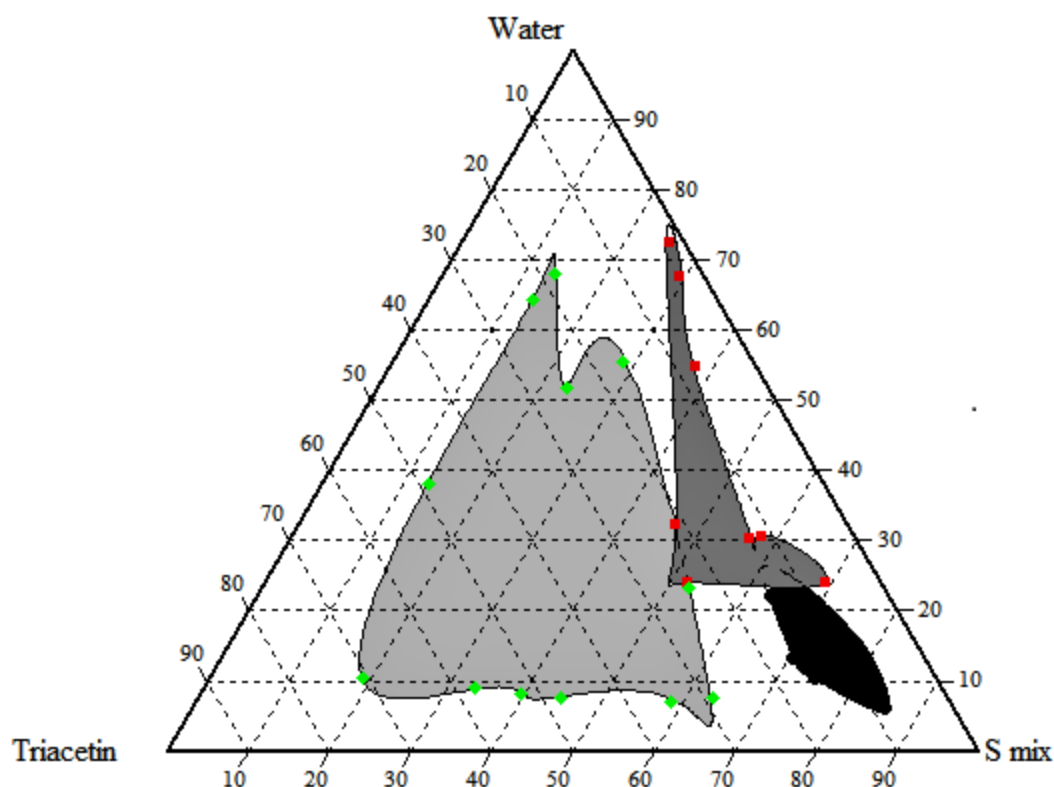


Figure 3. Pseudo ternary phase diagram of microemulsion.

Light gray: turbidity; gray: NE; and black: gel.

Characterization of extract loaded NE

A lower diameter of the drops of oil (<100 nm) (Tenjarla, 1999) and the transparency of the system confirm the presence of NE. DLS evidences a homogeneous system, with a narrow size distribution for all samples, low PDI and mean diameter values. Empty NE shows an average diameter of 11.50 ± 0.16 nm and PDI 0.15 ± 0.02 ; extract loaded NE has sizes of 11.82 ± 0.13 and PDI 0.12 ± 0.02 . The dimensional data were compared with those obtained by TEM analysis, which confirmed the presence of dispersed droplets with size less 20 nm (Figure 4).

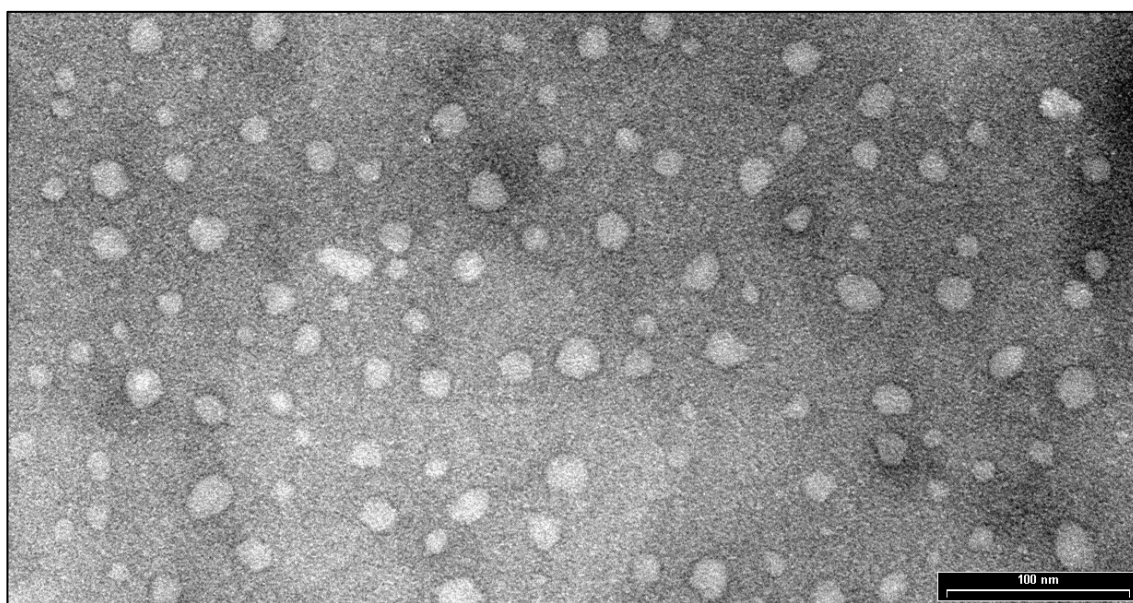


Figure 4. TEM image of VAC extract-loaded NE

Scale 100 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Solubility of VAC extract into NE

The solubility of the extract into NE was determined by introducing increasing amounts of extract in the formulation (Table 3). The samples were analyzed by HPLC, after 48 h under magnetic stirring at RT. NE improved the solubility of the extract about 10 times, compared to that in water. The extract dissolves entirely in the NE until 60 mg/ml, while its aqueous solubility results less than 6 mg. Table 3 reported the quantity of constituents solubilized into NE with an increasing amount of added VAC extract. The solubility of both classes of constituents was enhanced about 10 folds.

Sample	Total flavonoids (mg/mL)	Total iridoids (mg/mL)
5 mg/ml of extract	0.26	0.11
10 mg/ml of extract	0.41	0.16
15 mg/ml of extract	0.75	0.26
40 mg/ml of extract	2.41	0.60
60 mg/ml of extract	3.47	1.75
Water	0.31	0.17

Table 3. Solubility of VAC extract into NE.

Stability study

Empty and extract-loaded NEs were stored away from light at 4°C for approximately 2 months, in order to determine their stability. The physical stability was measured by monitoring the size of dispersed phase by using DLS; chemical stability was assessed by quantifying total flavonoids and iridoids. Furthermore, for each time point, macroscopic observations were performed to check the transparency of the sample and the occurrence of possible coalescence, creaming and separation phenomena.

Based on the visual identification, empty or extract-loaded NEs remained transparent for 2 months without the occurrence of phase separation at 4°C. Furthermore, NEs can be diluted with an aqueous solution without destroying their structure for 24 h. This aspect is essential to define the solubilization capacity because NE will be diluted by water in the gastrointestinal tract upon oral administration, with possible drug precipitation. Furthermore, it is a confirmation that we are dealing with a NE and not with micelles that would collapse with the dilution.

Empty NE showed an excellent physical stability: it remained transparent and the droplet size (11.50 ± 0.16) and PDI (0.15 ± 0.03) were unaffected. The select formulation was also stable at room temperature for 30 days, without showing phase separations or changes in the dimensions of the dispersed phase.

NE containing VAC extract also showed good physical stability for 60 days: it remained transparent, homogenous with constant droplet size (from 11.82 nm and PDI 0.12 to 11.20 nm and PDI 0.14).

Figure 5 evidenced the chemical stability of flavonoids and iridoids. As evidenced from the results, the stability of formulation is limited to 34 days, after which there is a partial degradation of both classes of constituents. Furthermore, in an effort to mimic physiological dilution process after oral administration, the NE was diluted up to 20-folds in simulated intestinal fluid and simulated gastric fluid. DLS analyses confirmed the physical stability of the system.

In particular, mean diameters and polydispersity values resulted unmodified at tested pHs in comparison with empty formulation (not diluted or diluted up to 20-folds in water, pH 5.5). Indeed, at pH 1.2 the sizes were 11.82 ± 0.13 nm and PdI 0.12 ± 0.02 ; at pH 5.5 the sizes were 11.75 ± 0.28 nm with PdI 0.22 ± 0.03 , and at pH 6.8 the sizes resulted 11.99 ± 0.54 nm and PdI 0.21 ± 0.03 . Values are the mean $\pm$ S.D. of three independent experiments.

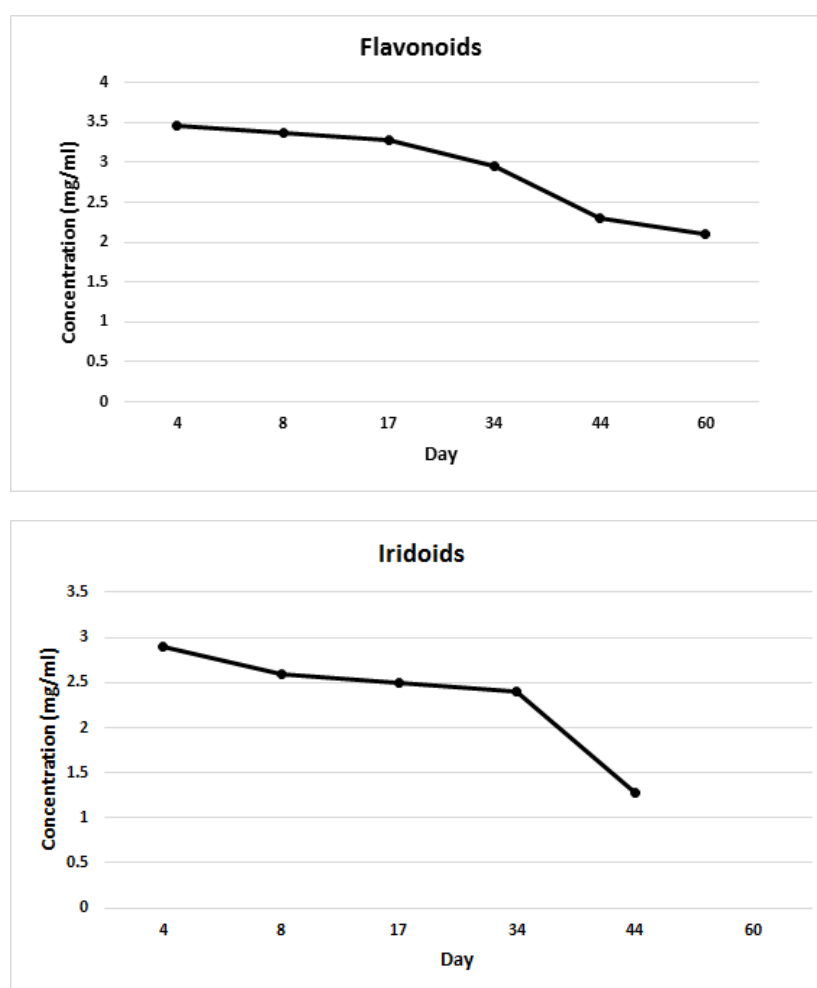


Figure 5. Chemical stability of flavonoids and iridoids of VAC extract formulated into NE after storage for 2 months.

Mean $\pm$ SD; n=3.

In vitro parallel artificial membrane permeability assay (PAMPA)

PAMPA assay is a method for predicting passive intestinal absorption (Kansy et al., 1998). PAMPA test is robust and reproducible and relatively fast (4-16 h) (Hiremath et al., 2009; Righeschi et al., 2016). In the last decades, it resulted as valuable complement and alternative to Caco-2 assay in the pharmaceutical research (Bermejo et al., 2004; Kerns et al., 2004; Sandhya et al., 2008). The experiment was carried out measuring the ability of VAC constituents to diffuse from a donor compartment, containing NE formulation, to acceptor compartment, through a PVDF membrane. NE provided improved permeability of both flavonoids and iridoid agnuside after 4 h, in comparison with a saturated aqueous solution used as control (Figure 6). This fact is undoubtedly due to the high solubilizing effect of NE on the constituents of extract, as previously reported in Table 3. The high amount of extract solubilized in the donor compartment promotes an increased passage through the membrane in the acceptor compartment.

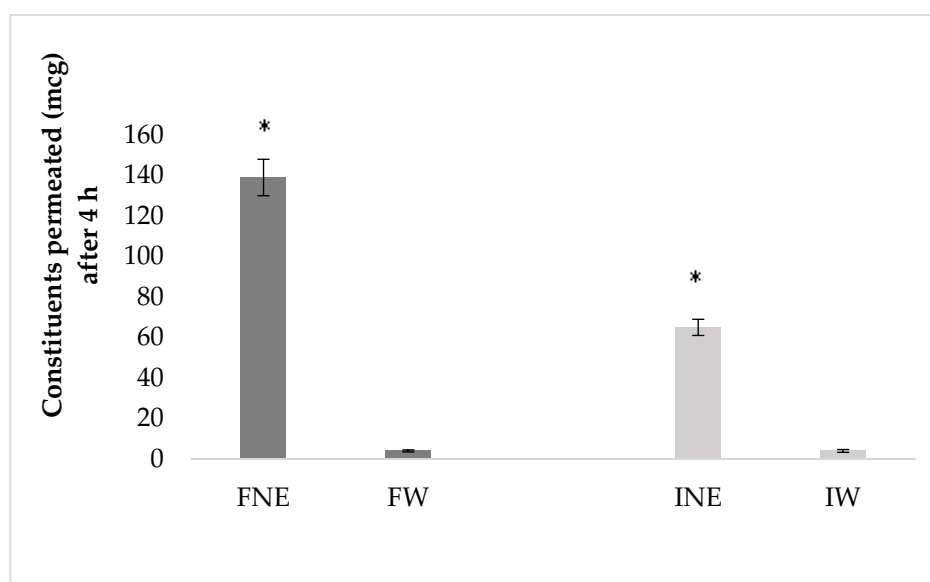


Figure 6. Comparative PAMPA permeability: micrograms of total flavonoids (F) and iridoids (I) of extract of VAC permeated from nanoemulsion (NE) and from saturated aqueous solution (W).

Average values $\pm$ S.D. of experiments carried out in triplicate are presented. * $p < 0.05$ vs water

The effective permeability (P_e) of flavonoids and iridoids were calculated as reported in the literature (Chen et al., 2008; Hiremath et al., 2009). P_e was $46.5 \pm 5.2 \times 10^{-6}$ cm/sec for flavonoids and $41.3 \pm 6.5 \times 10^{-6}$ cm/sec for iridoid. In the case of aqueous solution P_e were $14.1 \pm 1.2 \times 10^{-6}$ cm/sec for flavonoids and $29.5 \pm 2.4 \times 10^{-6}$ cm/sec for iridoids. The values confirmed that NE also increased the permeability of the extract compared to the aqueous solution.

Caco-2 permeability study

The Caco-2 monolayer was used as a model to study drug absorption across the intestinal epithelium. This well-differentiated human intestinal epithelial cell line is cultivated for studies of the transepithelial transport of drugs. In general, the aim is to investigate if a drug is actively or passively transported across the intestinal epithelium and, in the case of active mechanism, to identify the relevant carrier (Yee 1997; Artursson et al., 2001).

Caco-2 cells are most widely and successfully used permeation model, that also allows to investigate paracellular permeability and active efflux (Balimane et al., 2006). The potential cytotoxicity of VAC-loaded NE on Caco-2 cells were tested in order to find the highest non/low toxic concentrations to be used in transport experiments. The percentage of cell death after exposure to vitex NE for 24 h at the final concentration of 1:600–1:800 was negligible compared to control (untreated cells). We also tested concentrations from 1:100–1:300 for short exposure time (f 4-h). With this approach, a low to moderate cytotoxicity ranging from 5 to 20 % was observed. However, in order to overcome the limits of detection of HPLC analysis, the concentration of 1:100 was considered acceptable for transport experiments.

NE provided enhanced flavonoids and iridoids permeability in Caco-2 model, as evidenced in Figure 7. The apparent permeability values (P_{app}) was $25.0 \pm 3.4 \times 10^{-6}$ cm/sec for flavonoids and $24.0 \pm 1.8 \times 10^{-6}$ cm/sec for iridoids.

Thus, the flavonoids and iridoids in the NE exhibited the P_{app} values, necessary for complete intestinal absorption in humans (Artursson and Karlsson, 1991). Whereas, the VAC extract has not the permeability characteristics that could ensure this process. In the case of the aqueous solution, only flavonoids (isovitexin and casticin) resulted detectable into acceptor compartment after 4 h with a P_{app} corresponding to $8.1 \pm 0.9 \times 10^{-6}$ cm/sec. Agnuside are not detectable after 2 h, while its P_{app} resulted $10.3 \pm 2.1 \times 10^{-6}$ cm/sec after 4 h. The recovery of flavonoids and iridoids was above 85% respectively, suggesting that the obtained P_{app} values were reliable. A recovery above 80% is required for an acceptable *in vitro* prediction of P_{app} values (Hubatsch et al., 2007).

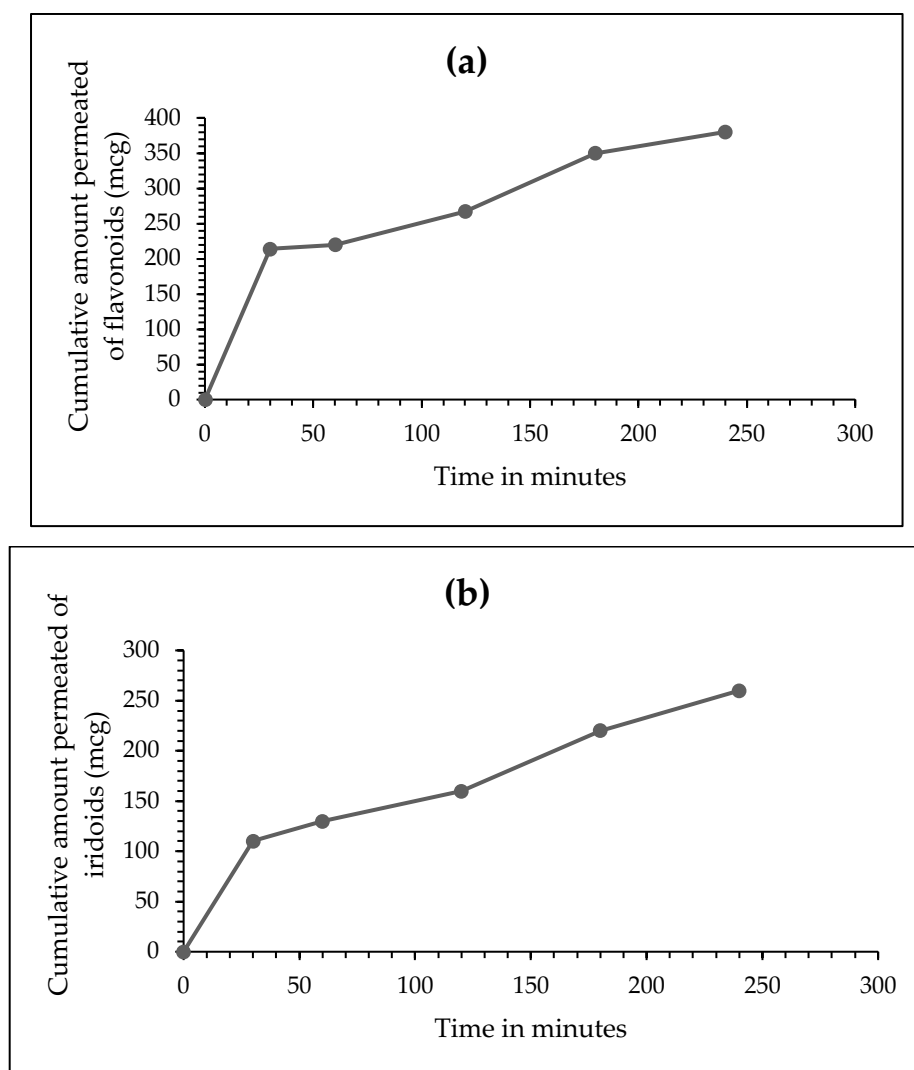


Figure 7. Caco-2 permeability of flavonoids **(a)** and iridoids **(b)** loaded into NE. Mean $\pm$ SD; n=3.

There was a good correlation between PAMPA and Caco-2 permeability results. Both models showed that the permeation of extract resulted improved when it is formulated in NE. Good correlation between PAMPA and Caco-2 model indicated that the passive diffusion might be an important mechanism for permeation. As previously reported, PAMPA and Caco-2 permeation data have a good correlation to human oral absorption (Kerns et al., 2004). Given the characteristics of the two methods, these assays can be applied synergistically for efficient and rapid investigation of permeation mechanisms in preformulation studies, not only in the case of single molecules but also for complex matrices, such as extracts and their formulations, as reported in this study.

CONCLUSIONS

The VAC extract was formulated into NE. This formulation provides enhanced dissolution of the main constituents the extract. The physical nature of these systems, the mechanism of drug entrapment, as well as the physicochemical interactions of constituents determine their substances solubilization capacity and physical stability during storage and upon dilution.

The optimized formulation was obtained with triacetin as the oil phase, Labrasol and Cremophor EL as surfactant and co-surfactant, respectively. It resulted in an appropriate formulation concerning of size, polydispersity, encapsulation efficiency and its positive influence on the solubility of extract (60 mg/ml).

NE can be diluted with aqueous buffer and it was stable for at least two months at 4°C.

The *in vitro* transport studies, PAMPA and Caco-2 models, revealed that the optimized formulation was successful in enhancing the permeation both of flavonoids and iridoids, with respect an aqueous solution. The increase of *in vitro* permeation appears to be due to the effect on improved solubility/dissolution of extract. After oral administration, the solubility and gastrointestinal permeability are fundamental parameters that control the rate and the extent of absorption and bioavailability. Consequently, *in vitro* dissolution has been recognized as an essential stage in drug development; hence, the improvement of the dissolution rate of poorly soluble drug and of their bioavailability is a great challenge to pharmaceutical scientists. PAMPA and Caco-2 can synergistically provide invaluable information about the permeability of single molecules and more complex substrates, such as extract and formulations. PAMPA assay is a particularly helpful complement to cellular permeability models, such as Caco-2, for its speed, low cost and versatility.

Thus, from the *in vitro* results, NE proved to be a promising oral delivery system for enhancing solubility and absorption of VAC extract.

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Caco-2 cells studies were performed in collaboration with Dr. Cristina Luceri and Dr. Elisabetta Bigagli (NEUROFARBA, Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).



Drug Delivery



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Nanoemulsion for improving solubility and permeability of Vitex agnus-castus extract: formulation and in vitro evaluation using PAMPA and Caco-2 approaches

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**PREDICTION OF PERMEATION AND CELLULAR TRANSPORT OF
SILYBUM MARIANUM EXTRACT FORMULATED IN A NANOEMULSION
BY USING PAMPA AND CACO-2 CELL MODELS**

INTRODUCTION

Silybum marianum L. Gaertn. (SM, milk thistle) is an annual or biennial plant of the Asteraceae family. Historically, it was used to treat disorders of the liver, spleen, and gallbladder. The main group of active constituents of SM seeds, known as silymarin, consists of flavonolignans (silybin, isosilybin, silycristin and silydianin, Figure 1) and taxifolin, a flavanolol (ESCOP, 2009).

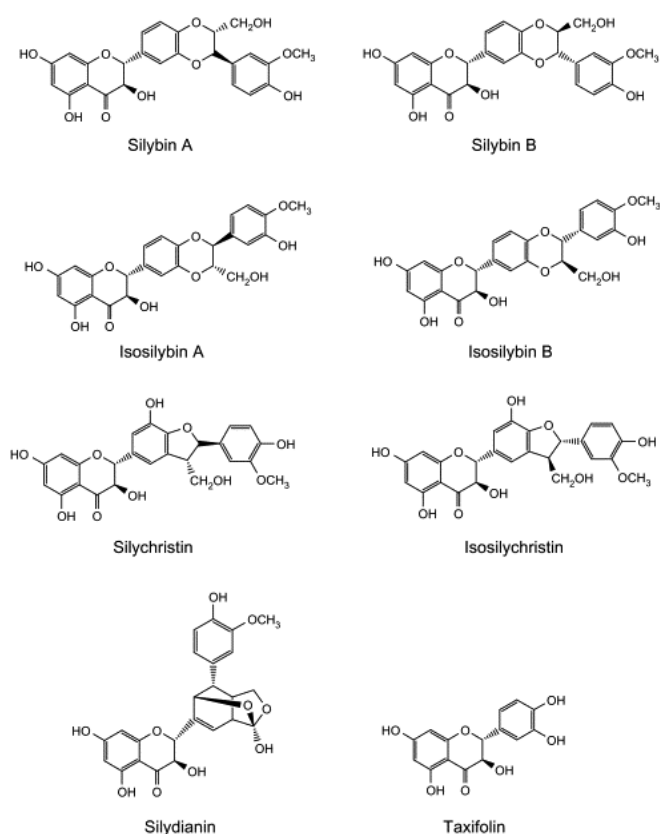


Figure 1. Chemical structures of the main constituents of silymarin.

They have a broad spectrum of bioactivities and pharmacological activities. Well known as a hepatoprotector, silymarin is effective clinically to treat a variety of liver disorders, (O'Hara et al., 1998; Pepping, 1999), certain cancers, such as breast, prostate and skin cancers (Zi, Feyes et al., 1998; Zi, Grasso et al., 1998).

Silymarin has been found to be beneficial in type 2 diabetes patients and many articles demonstrated decreases in both fasting and mean daily glucose, triglycerides and total cholesterol levels (Velussi et al., 1997; Lirussi et al., 2002; Huseini et al., 2006). However, the effectiveness of silymarin was discounted by its poor water solubility and low bioavailability after oral administration (Pepping, 1999). Orally administered silymarin is absorbed rapidly with a $t_{\max}$ of about 2-4 h and a $t_{1/2}$ of 6 h (Pepping, 1999). Only 20-50% of the extract is absorbed after oral administration and undergoes extensive enterohepatic circulation (Morazzoni et al., 1993).

Our main purpose is to search for technological strategies to overcome all the disadvantages associated with conventional formulations, in particular, to improve bioavailability after oral administration and consequently the therapeutic efficacy.

Many approaches were employed in order to improve the efficacy and bioavailability of silymarin or silybin, such as cyclodextrin inclusion complexes (Arcari et al., 1992), solid dispersion pellets (Sun et al., 2008), proliposomes (Xiao et al., 2006), self-microemulsifying drug delivery systems (Wu et al., 2006; Woo et al., 2007), nanoemulsions (Parveen et al., 2011), phospholipid complexes (Xiao et al., 2005).

The study is aimed at developing a new oral formulation, which can overcome the limited oral bioavailability increasing the therapeutic efficacy of a SM commercial extract. Oil in water nanoemulsion (NE) was formulated with food acceptable components, to increase solubility, stability and ameliorate intestinal permeability of extract's constituents.

In recent years, lipid-based formulations have been used to improve the oral bioavailability of poorly water-soluble compounds. Lipid carriers promote absorption by presenting the drug to the gastrointestinal tract in a solubilized form, increasing the solubilization capacity of the gastrointestinal fluids, and, in many cases, generating transiently supersaturated drug concentrations (Porter et al., 2007; Porter et al., 2008). NEs have attracted much interest being highly dispersed, stable and transparent systems and easy to prepare. Furthermore, their nanoscopic dimensions allow a better absorption by the cell membranes. Finally, NEs show the ability to solve the problems of solubility and stability of many phytotherapeutic, nutraceuticals and food additive (Bergonzi et al., 2014), because they avoid the dissolution step.

After qualitative and quantitative HPLC-DAD characterization of commercial SM extract, it was incorporated into selected oil-in-water NE and its physical/chemical stabilities and release properties were investigated. NE was physically and chemically analyzed with DLS, TEM and HPLC-DAD analyses to evaluate size, homogeneity, morphology and encapsulation efficiency. Stability in simulated gastric fluid, followed by simulated intestinal conditions was also considered.

In vitro permeation or transport studies using different models such as parallel artificial membrane permeability assay (PAMPA) (Kansy et al., 1998) and Caco-2 cell line were also evaluated to determine the suitability of the prepared NE for oral delivery.

Based on the characteristics of the two methods, these assays can be synergistically applied to an efficient and rapid investigation of permeation mechanisms in pre-formulation studies, not only in the case of single molecules but also for complex matrices, such as extracts and their formulations, as reported in this study.

MATERIALS

Silybum marianum dry extract, was supplied by Bionorica SE, Neumarkt, Germany. The dry extracted complies with the monograph *Silybi mariani extractum siccum raffinatum et normatum* (Ph. Eur. 8.0/2071) of the European Pharmacopoeia. The DER is 24-27:1 and the extraction solvent is Acetone. Storage conditions: tightly closed, dry, protected from light, 15-25°C.

Taxifolin (purity $\geq 99\%$) was from Extrasynthese (Genay Cedex, France); silycristin (purity $\geq 95\%$) and silybin (purity $\geq 98\%$) were from Sigma-Aldrich (Milan, Italy).

Cremophor EL (Castor Oil Fatty Acids, Ethoxylated Glycerol Esters), Triacetin, ethanol analytical reagent, Acetonitrile and Methanol HPLC grade, Formic acid ($\geq 98\%$), Cholesterol, Lecithin, Dichloromethane, DMSO, 1,7-Octadiene ($\geq 98\%$), Phosphate Buffered Saline (PBS) bioperformance certified, Lipase from porcine pancreas, Pepsin from porcine gastric mucose, Bile salts, HCl were purchased from Sigma-Aldrich (Milan, Italy); Oleic acid was from Farmitalia, Carlo Erba SpA (Milan, Italy); Labrasol ECH (Caprylocaproyl polyoxyl-8 glycerides), Capryol 90 (Propylene glycol monocaprylate), Transcutol HP (Diethylene glycol monoethyl ether) were kindly provided from Gattefossé (Saint Priest, France).

Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, USA).

METHODS

Preparation of SM extract sample for HPLC-DAD analysis

SM commercial extract (500 mg accurately weight) was suspended in 20 mL of methanol, sonicated in an ultrasonic bath for 30 min, and filtered. The extraction was repeated three times. The filtrate was evaporated until dryness. Then, the residue was dissolved in methanol to prepare the sample for HPLC-DAD analysis. Aqueous solubility was determined by dissolving the extract in deionized water until saturation at room temperature. The undissolved solid was eliminated by centrifugation and the solution was analyzed by HPLC.

Preparation of standard solutions

Standard solutions were freshly prepared by dissolution of standard compounds in methanol to obtain a final concentration of 0.5 mg/mL.

Linearity range of response was determined for taxifolin, silycristin, silybin. All the calibration curves had coefficients of linear correlation $R^2 \geq 0.999$.

Limit of detection (LOD) and limit of quantification (LOQ) were determined by calculation of the signal-to-noise ratio. LOD resulted 5.05 ng for taxifolin, 6.9 ng for silycristin and 7.5 ng for silybin. LOQ resulted 12.2 ng for taxifolin, 11.7 ng for silycristin and 10 ng for silybin.

HPLC-DAD analysis

The separation of SLM extract constituents was carried out using an HP 1100 liquid chromatograph equipped with a DAD detector. A 150 mm x 4.6 mm i.d., 5 μ m Zorbax Eclipse XDB, RP18 column was used (all from Agilent Technologies, Santa Clara, CA, USA). The mobile phases were: (A) formic acid/water pH 3.2, (B) CH₃CN and (C) CH₃OH. Flow rate was 0.6 mL/min and the temperature was set to 26°C. The UV/vis spectra were recorded in the range 200-700 nm and the chromatograms were acquired at 210, 280, 350 nm.

The following solvent gradient was applied: 0-10 min, 63-58% A, 15-20% B, 22-22% C; 10-15 min, 58-30% A, 20-30% B, 22-40% C; 15-20 min 30-30% A, 30-30% B, 40-40% C; 20-25 min, 30-63% A, 30-15% B, 40-22% C with equilibration time of 5 min.

Solubility studies

The extract's solubility in different vehicles was measured to find an appropriate medium, with a good solubilizing capacity of SLM extract and useful either as lipophilic phase or co-surfactant. An excess amount of SLM extract was added to 5 mL of each selected solvent (triacetin, tocopherol acetate, oleic acid, Labrafil, Capryol 90, Cremophor EL, Labrasol ECH, Transcutol HP and Labrafac PG ECH, water). Each mixture was shaken at 25°C for 24 h and then was centrifugated at 13148 x g for 10 min. The concentration of the components of the extract was determined by HPLC after dilution with methanol/dichloromethane (3:2 v/v). The analyses were performed in triplicate.

Construction of ternary phase diagram

Pseudoternary phase diagrams were constructed to obtain the concentration range of all components in which they form a NE. The pseudoternary phase diagrams were constructed using the water titration method. Surfactant and co-surfactant were mixed at different weight ratios (S_{mix}). For each S_{mix} ratio, a pseudoternary phase diagram was elaborated by testing weight ratio of oil/ S_{mix} of 0:100, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. Each oil- S_{mix} mixture was diluted under vigorous stirring dropwise with water. After equilibrium each sample was visually checked and the phase boundary was determined by observing the changes in the sample appearance, from turbid to transparent or from transparent to turbid, and by evaluating if nanoemulsion, emulsion or gel was present.

Solubility of SLM extract into NE

The ability of NE to solubilize the extract was investigated and compared with respective aqueous, micellar solution of surfactant or oil solution. In order to determine the maximum loading capacity of NE, extract loaded formulation was prepared by dissolving increasing amount of extract into the NE. The solution was stirred for 24 h at 25°C and protected from light.

Final NE was obtained by dissolving extract into NE (40 mg/mL) and stirring to form a clear and transparent dispersion. The resulting formulation was tightly sealed and stored at + 4°C temperature.

The quantity of constituents solubilized was defined by HPLC-DAD analysis at 280 nm. The analyses were performed in triplicate.

Particle size analysis and ζ -potential

Droplet size of the developed NE was measured by a Dynamic Light Scattering (DLS), Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25°C. Time correlation functions were processed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, Pdl) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the Cumulants method, fitting a single exponential to the correlation function to obtain particle size distribution. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle, diluting the samples in distilled water. ζ -potentials was measured using the same instrument; for all samples, an average of three measurements at stationary level was taken. The temperature was kept constant at 25°C by a Haake temperature controller. ζ -potential was calculated from the electrophoretic mobility, using the Henry correction to Smoluchowski's equation.

Morphological characterization

Morphology and structure of NE were studied using a transmission electron microscope (TEM, Jeol Jem 1010, Tokyo, Japan). 10 μ L of NE after appropriate dilution was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen, which was stained with a phosphotungstic acid solution 1% w/v in sterile water. The samples were dried for 3 min and then were examined under a JEOL 1010 electron microscope and photographed at an accelerating voltage of 64 kV.

In vitro release studies

The dialysis bag method was applied to study the release of SLM from NE using a mixture of CaCl₂ 750 mM (pH 7): EtOH 60:40, as dissolution medium. The release was monitored for 24 h. The dialysis bags were hydrated before use. The bag containing 1 mL of NE was placed in a becker containing 200 mL of dissolution medium maintained at 37 °C ± 0.5 °C, under magnetic stirring. At different time intervals, aliquots of the dissolution medium were withdrawn and replaced with the same volume of fresh medium. The samples were suitably diluted and analyzed by HPLC-DAD for quantification of constituents of the SLM extract. All the operations were carried out in triplicate.

The percentage of silymarin released in the medium at pH 7 at each point time was calculated by applying the following formula:

$$\% \text{ silymarin released} = (\text{mg silymarin}_t \times 100) / \text{mg silymarin}_{\text{tot}}$$

Where:

mg silymarin_t = mg released for each time point *t*

mg silymarin tot = total mg of silymarin loaded in NE.

Stability studies

The samples were inserted into sealed glass vials and stored at 4°C for 40 days and at room temperature (25°C) for three months to evaluate the stability of empty and extract-loaded NEs. Chemical and physical stabilities were assessed by monitoring the occurrence of phase separation, dispersed phase size and drug content at predetermined intervals by DLS and HPLC-DAD analyses.

Furthermore, the samples were diluted 10, 20 and 30-fold with distilled water to mimic physiological dilution process after oral administration. The dilutions were followed by gentle vortexing for 2 min at room temperature. The intragastric stability was tested in simulated gastric fluid (SGF) (Aditya et al., 2013). Briefly, 5 mL of NE were suspended in 5 mL of SGF (0.32% w/v pepsin, 2 g of sodium chloride and 7 mL HCl dissolved in 1 L water and pH adjusted to 1.8 using 1M HCl) and incubated at 37°C under shaking. After 2 h, the sample was collected to analyze the size and Pdl.

After digestion in simulated stomach condition, previously sample was subjected to digestion under simulated intestinal condition containing intestinal enzyme complex (lipase 0.4 mg/mL, bile salts 0.7 mg/mL, and pancreatin 0.5 mg/mL) at pH 7.0, 37 °C under shaking (Aditya et al., 2013). After 2 h digestion in simulated intestinal fluid (SIF), the sample was collected and its physical stability was checked by DLS analysis.

In vitro parallel artificial membrane permeability assay (PAMPA)

The assay was carried out in a 96-well, MultiScreen-IP PAMPA (Millipore Corporation, Tullagreen, Carrigtwohill, County Cork, Ireland) filter plate. The ability of compounds to diffuse from a donor compartment, through a PVDF membrane filter pretreated with a lipid-containing organic solvent, into an acceptor compartment is evaluated. 5 μ L of lecithin (10 g/L) and cholesterol (8 g/L) in 1,7-octadiene solution were added to the filter of each well. Immediately after the application of the artificial membrane, 250 μ L of drug-containing samples (saturated solution of extract in water and SLM loaded NE, 40 mg/mL) were added to each well of the donor plate. 250 μ L of buffer (0.05 mL/mL DMSO/PBS, pH 7.4) were added to each well of the acceptor plate. The donor plate was then placed into acceptor the plate, ensuring that the underside of the membrane was in contact with buffer. The plate was covered and incubated at room temperature under shaking for 6 hours and permeation was evaluated at 0.5, 2, 4 and 6 hours.

After incubation, 6 hours, the content silymarin and taxifolin into donor and receptor compartment were analyzed by HPLC-DAD. The experiment was performed in triplicate and mean of three samples was used in the data analysis. Permeability of the compounds was calculated using the following formula (Chen et al., 2008):

$$P_e = -\ln[1 - C_A(t)/C_{\text{equilibrium}}]/A \times (1/V_D + 1/V_A) \times t$$

where P_e is permeability in the unit of cm/s. A = effective filter area = $f \times 0.3 \text{ cm}^2$, where f = apparent porosity of the filter, V_D = donor well volume = 0.25 mL, V_A = receptor well volume = 0.25 mL, t = incubation time (s), $C_A(t)$ = compound concentration in receptor well at time t , $C_D(t)$ = compound concentration in donor well at time t , and

$$C_{\text{equilibrium}} = [C_D(t) \times V_D + C_A(t) \times V_A] / (V_D + V_A)$$

Cell Culture

The human colon carcinoma cell line Caco-2 was kindly provided by Prof. Masini (University of Florence, Italy). Cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. Caco-2 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Upon reaching 80% confluence, cells were sub-cultured weekly at a split ratio of 1:3 by trypsinization.

MTS assay for cell viability

Viability analysis was performed using Cell Titer 96 Aqueous One solution cell proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay kit (Promega Madison, WI, USA). In brief, Caco-2 cells were transferred to flat bottom 96-well tissue culture plates (Corning, USA) at a seeding density of 5×10^3 cells/well and allowed to grow for 24 h under the conditions detailed above. For MTS assay, the culture medium was removed and replaced with fresh medium containing SM loaded NE and the cells were incubated for a further 4 or 24 h. The cells were then exposed to MTS solution and allowed to incubate for 2 h at 37 °C. The product of the reaction was measured at 490 nm using a spectrophotometer (Multilabel Counter 1240 Victor 3, Perkin Elmer). Cell death was expressed as a percentage of values obtained from control, untreated cells calculated from three replicates of each NE dilution.

Cell culture for Transport Studies

For transport studies, cells were seeded at 50000 cells/well in cell culture inserts with PET (polyethylene terephthalate) membranes (0.4 μ m pore size, 0.33 cm² growth surface area; BRAND, Italy). Culture medium (DMEM) was added to apical (AP) (0.5 mL) and basolateral (BL) (1.5 mL) side and was replaced every other day for the first week and daily after that. Cells were let to differentiate for 18-21 days.

Monolayer integrity

The integrity of the layer was evaluated with the lucifer yellow (LY) permeability assay according to Iacomino et al., 2013. LY was diluted in transport buffer (HBSS with Ca^{2+} , Mg^{2+} , 25 mM HEPES, pH 7.4) and added to the AP compartment at a final concentration of 100 μM . After incubation at 37°C for 1 h, the HBSS in the BL chamber was collected, and the concentration of LY was determined by using 485 nm excitation and 530 nm emission on a fluorescence plate reader (Multilabel Counter 1240 Victor 3, Perkin Elmer). The percentage of AP to BL permeability was calculated according to the following equation:

$$\% \text{ permeability} = (\text{Fluorescence in the BL-blank})/(\text{Fluorescence LY-blank}) \times 100$$

The maximum critical flux of LY to identify leaky monolayers was estimated to be less than 3% of starting concentration.

Transport experiments

Transport study was performed according to Hubatsch et al., 2007. Before the transport study, the culture medium (DMEM) was replaced with preheated (37°C) transport HBSS medium supplemented with 25 mM HEPES (pH 7.4). After the cell monolayer was equilibrated for 30 min at 37 °C, Caco-2 cells were treated for 4 h with different dilutions of SM-loaded NE in HBSS in the AP chamber, while the BL chamber contained only HBSS.

For the test, 0.5 mL of aqueous saturated solution of extract or NE diluted 40 folds with culture medium was added to AP side, while BL side was filled with culture medium. At predetermined intervals (30 min, 1, 2, 4 h) 0.3 mL of medium in the BL side was taken for HPLC analyses and replaced with the same volume of fresh HBSS. At the end of the experiments, the integrity of the layer was re-evaluated with the LY permeability assay as described above.

Apparent permeability coefficients (P_{app}) were calculated according to the following formula:

$$P_{app} = \frac{\Delta Q / \Delta t}{C_0 \times A}$$

where $\Delta Q / \Delta t$ indicates linear appearance rate of mass in the basolateral side; C_0 , initial concentration in apical side; and A , surface area (i.e., 0.33 cm²).

Recovery in PAMPA and Caco-2 tests

The recovery (mass balance) is defined as the sum of the drug recovered from the acceptor chamber and the drug remaining in the donor chamber at the end of the experiment, divided by the initial donor amount. Recovery for SLM and taxifolin was calculated according to the equation:

$$\text{Recovery (\%)} = C_{Df} V_D + C_{Rf} V_R / (C_{D0} V_D) \times 100$$

where C_{Df} and C_{Rf} are the final concentrations of the compound in the donor and receiver compartments, respectively, C_{D0} is the initial concentration in the donor compartment, and V_D and V_R are the volumes in the donor and receiver compartments, respectively. All results are expressed as means $\pm$ SD.

Statistical analysis

Experiments were repeated three times and results expressed as a mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

HPLC-DAD analysis

An HPLC-DAD method was developed to the quali-quantitative characterization of the SM commercial extract. The wavelengths of 210, 280, 350 nm were selected for acquiring main constituents of extract. In order to achieve better chromatographic separation, various linear gradients of binary or ternary mixture acetonitrile, water and methanol were investigated at different flow-rates, by using various RP-18 columns. Finally, the gradient program described in the experimental part was selected for its separative efficiency. The chromatographic profile of the methanolic extract at 280 nm is reported in Figure 2. The identification of compounds was carried out by UV absorption spectra and by comparison with standard compounds and literature data (Shibano et al., 2006; Quaglia et al., 1999; Wang et al., 2010). The chromatographic conditions allowed a good separation of two diastereoisomers of silybin and silychristin and the peaks related to taxifolin and isosilybin (Figure 2). The dry commercial extract contains 55.24% w/w of silymarin and 1.88% w/w of taxifolin, according to literature (ESCOP, 2009).

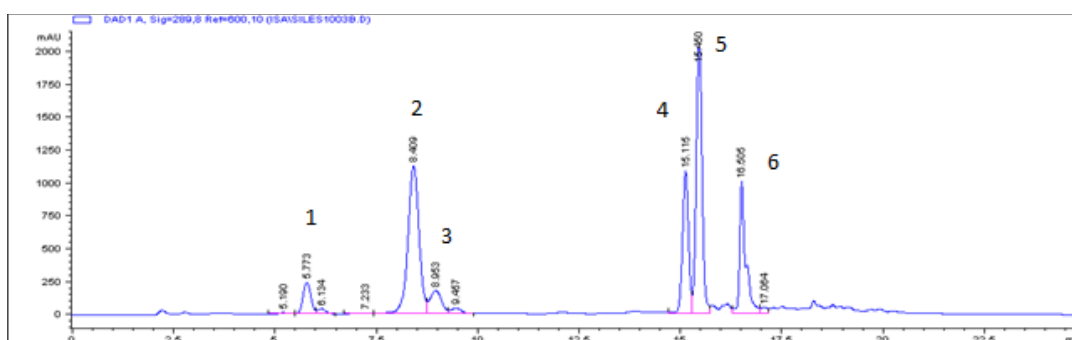


Figure 2. HPLC-DAD profile of *Silybum marianum* extract at 280 nm.

1. 5.77 min taxifolin; 2. 8.41 min silychristin A; 3. 8.96 min silychristin B; 4. 15.11 min silybin A; 5. 15.45 min silybin B; 6. 16.50 min isosilybin.

Solubility studies

The solubility of SM extract was determined in various oils, surfactants and cosurfactants to find out appropriate components of NE (Table 1). Selected vehicles show a different influence on the solubility of the constituents, compared to water. All vehicles increase the solubility of both silymarin and taxifolin.

	Taxifolin (mg/mL)	Silychristin (mg/mL)	Silybin (mg/mL)	Isosilybin (mg/mL)
Triacetin	0.19 ± 0.05	0.35 ± 0.01	2.61 ± 0.08	0.52 ± 0.01
Labrafil	0.19 ± 0.04	0.27 ± 0.09	2.33 ± 0.51	0.42 ± 0.07
Oleic acid	0.080 ± 0.005	0.19 ± 0.02	1.98 ± 0.11	0.33 ± 0.04
Capryol 90	0.69 ± 0.02	1.66 ± 0.04	8.21 ± 0.14	2.02 ± 0.03
Labrasol ECH	2.86 ± 0.17	17.43 ± 0.21	37.87 ± 1.83	8.00 ± 0.23
Transcutol HP	5.95 ± 0.27	25.12 ± 0.93	79.29 ± 2.74	17.60 ± 0.64
Cremophor EL	0.92 ± 0.25	2.30 ± 0.95	12.09 ± 2.86	2.53 ± 0.61
Water	0.05 ± 0.01	0.10 ± 0.04	0.03 ± 0.01	0.01 ± 0.00

Table 1. Solubility of constituents of SM extract in different vehicles.

Data displayed as mean ± SD; n = 3.

Construction of ternary phase diagram

Based on the solubility results, good lipophilic phases resulted Transcutol HP and Labrasol. These were mixed in different ratios with pairs of surfactant/cosurfactant (S_{mix}). The solubilizing effect of Transcutol is well known, and it was used in several dosage forms because of its ability to solubilize many drugs and also silybin (Woo et al., 2007). Therefore, Labrasol was preferred in the preparation of our NE, to evaluate a new formulation.

The optimized NE was assessed for clarity and transparency by visual inspection. The best system was obtained by using Labrasol as oil and Cremophor EL/Labrafil as surfactant/co-surfactant system (S_{mix}), in 1.5:1 ratio. The selection of the ingredients was driven by consideration of their toxicity and by knowledge of their mechanism of action on the permeability through the gastro-intestinal tract. Labrasol, and to a lesser extend Cremophor, were shown to open tight junctions (Sha et al., 2005).

The existence region of each NE was obtained by phase diagram (Figure 3).

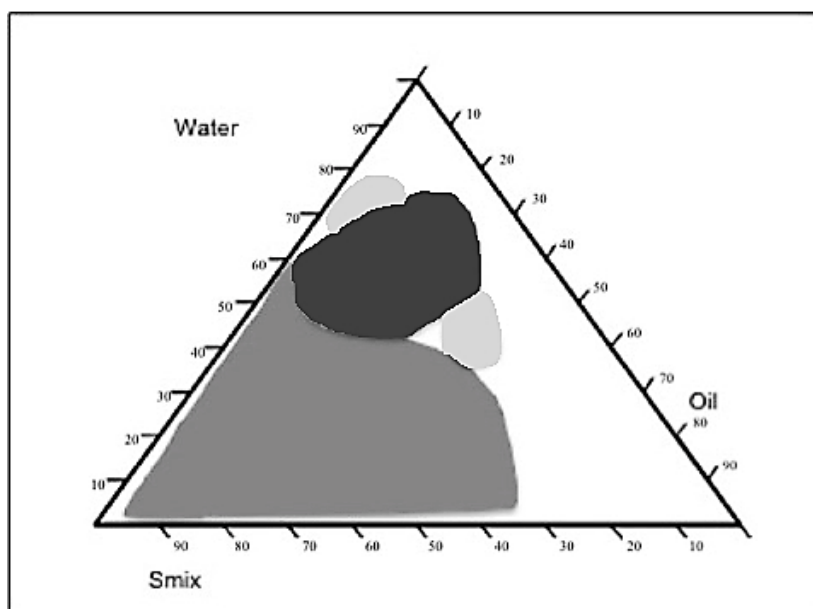


Figure 3. Pseudo-ternary phase diagram of NE.

Light gray: gel; dark gray: nanoemulsion; white: turbidity.

The pseudoternary phase diagram was built using the water titration method. Each sample was visually checked after equilibrium and determined as being clear nanoemulsion, emulsion or gel. Figure 3 shows the pseudo-ternary diagram with a field of the existence of selected NE (1:1 O/S_{mix} and S_{mix} 1.5:1). Finally, the composition resulted: 2.5 g of Labrasol (20%) as the oil phase, 1.5 g Cremophor EL as surfactant, 1 g of Labrafil as co-surfactant (S_{mix}, 20%) respectively. Deionized water was added drop by drop, under gentle agitation, to each oily mixture. Its final volume was 7.0 mL (60%).

Solubility of SLM extract into NE

The solubility of the extract into NE was also determined by adding increasing amounts of SM extract into NE. The samples were analyzed by HPLC, after 48 h under magnetic stirring at RT. The results were compared with data obtained with the saturated aqueous solution. NE improved the solubility of SM extract considerably compared to water: the extract was solubilized entirely at the dosage of 40 mg/mL, respect to its aqueous solubility, that results less than 0.3 mg/mL. The amounts of constituents solubilized into NE are reported in Table 2. A high increase of solubility was obtained for all constituents: the solubility of silymarin was ameliorated about 60 times.

	Taxifolin (mg/mL)	Silychristin (mg/mL)	Silybin (mg/mL)	Isosilybin (mg/mL)	Silymarin (mg/mL)
Water	0.05 ± 0.01	0.10 ± 0.04	0.03 ± 0.01	0.01 ± 0.00	0.13 ± 0.01
NE	0.34 ± 0.06	0.77 ± 0.03	5.71 ± 0.14	1.12 ± 0.05	7.60 ± 0.07

Table 2. Solubility of SM extract into NE *vs* water.

Data displayed as mean ± SD; n = 3.

Characterization of extract-loaded NE

Physical characterization of empty and extract-loaded NE confirmed the presence of a homogeneous system, with a narrow size distribution and low values of PDI (Table 3). These data were compared with those obtained by TEM analysis, which confirmed the presence of droplets with size less than 40 nm (Figure 4).

Thus, the developed NE represents a successful tool to incorporate SM commercial extract and to ameliorate its solubility significantly. It was suitable for oral administration in terms of physical characteristics and safety of constituents.

Sample	Size	PdI	ζ -potential
Empty NE	22.27 $\pm$ 1.08	0.11 $\pm$ 0.06	-1.1 $\pm$ 0.3 mV
NE (40 mg/mL of SM)	21.29 $\pm$ 0.41	0.11 $\pm$ 0.07	-5.1 $\pm$ 0.7 mV

Table 3. Technological characterization of empty SM extract loaded NE.

Data displayed as mean $\pm$ SD; n = 3.

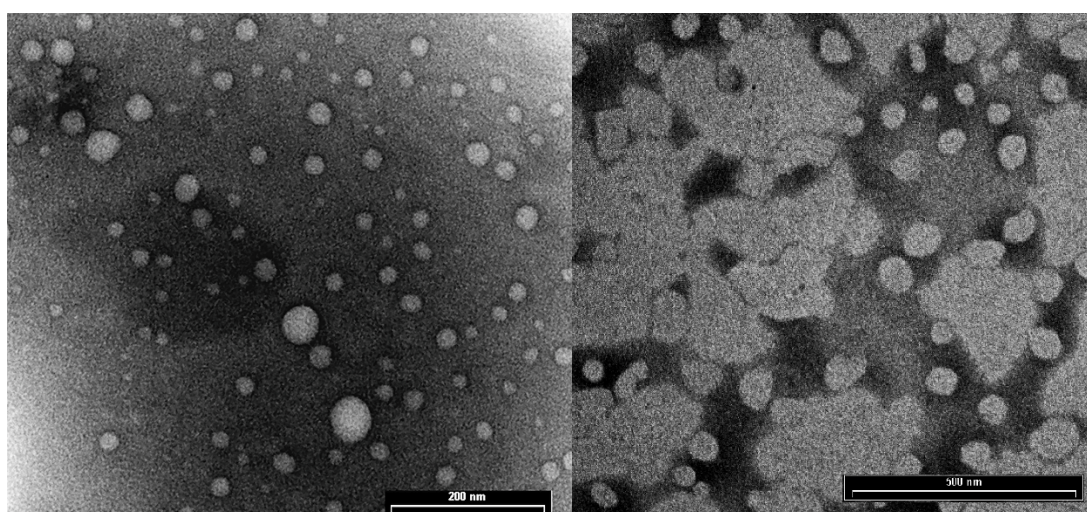


Figure 4. TEM images of empty (left) and SM extract loaded (right) NE.

Scale bar 200 and 500 nm, respectively.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Stability studies

Empty and extract-loaded formulations were stored away from light at +4 °C for 40 days to define their stability. Physical stability was assessed by monitoring the size of dispersed phase by DLS.

Extract-loaded NE resulted stable: not phase separation occurred, the size of droplets remains constant, ζ -potential ranged from -5.1 to -6.6 mV and PDI from 0.11 to 0.18. TEM results confirmed data obtained by DLS analyses: sizes range between 30 and 40 nm.

Chemical stability was obtained by quantifying the residual amount of main constituents (silybin, isosilybin, silychristin and taxifolin) of the extract, by using the HPLC-DAD method reported in the experimental part.

As evidenced in Figure 5, the concentration of silybin, isosilybin, silychristin and taxifolin remained constant during the whole period of the test. The developed formulation showed an excellent physical and chemical stability: size and polydispersity were unaffected, and no degradation of active constituents was observed during 40 days.

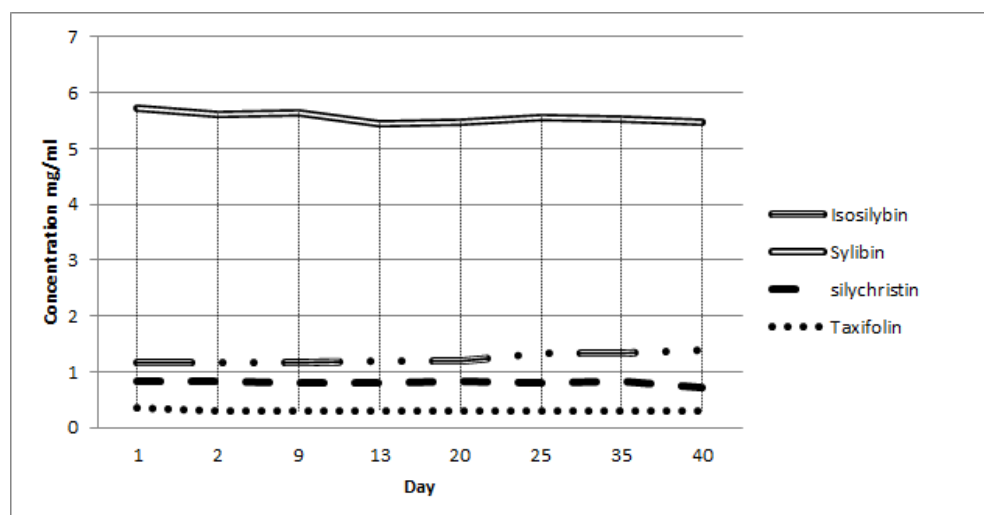


Figure 5. Chemical stability of the main constituents of the extract in the stability test at 4°C.
Data displayed as mean $\pm$ SD; n = 3.

The NE stability was also considered at room temperature for 3 months. No phase separation, creaming or cracking occurred, and the technological characteristic of disperse phase were unmodified (size of 20.09 ± 0.04 nm, PDI 0.06 ± 0.01 , ζ -potential -6.63 ± 1.73 mV).

The residual percentages of constituents remained unchanged (taxifolin: 0.31 ± 0.01 mg/mL; silybin: 5.73 ± 0.07 mg/mL; isosilybin: 1.23 ± 0.01 mg/mL; silychristin: 0.80 ± 0.05 mg/mL).

In vitro lipid digestion assay in a simulated gastrointestinal medium was also performed to evidence if NE could protect active constituents of extract from degradation during transit through an unfavourable environment in the gastrointestinal tract until the intestine. The intragastric stability was tested in simulated gastric fluid (SGF) in the presence of pepsin for 2 h, followed by treatment with simulated intestinal fluid (SIF) in the presence of the pancreatin-lipase-bile extract mixture for 2 h. Samples were collected and analyzed by DLS analysis to check their physical stability. The analyses confirmed the physical stability of the systems concerning size and homogeneity. NE was stable and no aggregation or degradation phenomena occurred. Sizes of the dispersed phase of NE in SGF (23.3 ± 3.5 nm) and SIF (23.0 ± 1.5 nm) media remained around 20 nm.

In vitro release studies

In vitro release study allowed to understand the kinetic release of extract loaded in NE for 24 hours. The dissolution medium was a mixture of CaCl₂ 750 mM: EtOH 60:40.

Figure 6 shows the release profile. A gradual release was found and the maximum percentage of silymarin obtained resulted $58.5\% \pm 2.7$ after 24 h. In this case, any released constituent is significantly diluted once it leaves the bag. Perhaps due to the large volume of the dissolution medium (200 mL) and the poor solubility in water of taxifolin, only silymarin can be quantified.

Furthermore, the kinetic was studied and the correlation coefficient (R^2 0.984) is in agreement with the kinetics of release of Hixson model (Dash, 2010).

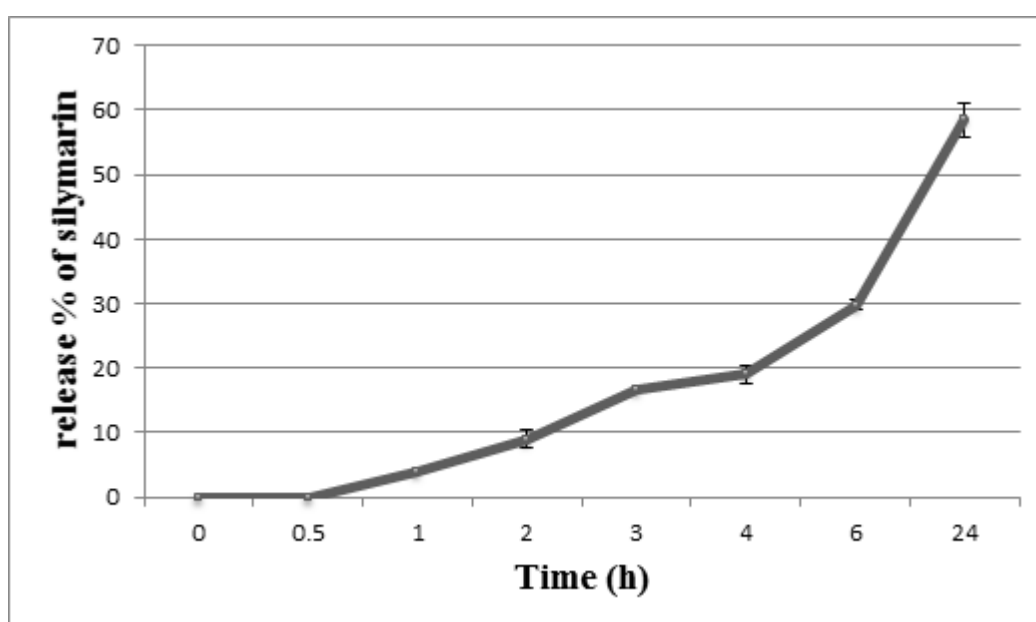


Figure 6. *In vitro* release profile of silymarin.

Data are the mean $\pm$ SD; n = 3.

In vitro parallel artificial membrane permeability assay (PAMPA)

The experiment was carried out measuring the ability of saturated aqueous solution of SM extract and extract loaded NE to diffuse from donor to acceptor compartment, through a membrane, to evaluate the influence of the formulation on the permeability of extract. By comparison of quantities permeated at different incubation time, we observed an increase of the amount of extract constituents permeated, in comparison with a saturated aqueous solution of extract, used as control Table 4. After 30 min the quantity of components permeated was negligible for both the samples. After 6 hours, the permeation increased for the extract formulated into NE.

Incubation time	Constituents	NE	Aqueous solution
2h	Silymarin	0.220 ± 0.006	0.023 ± 0.002
	Taxifolin	6.37×10^{-3}	0.4×10^{-3}
4h	Silymarin	0.570 ± 0.041	0.079 ± 0.002
	Taxifolin	0.028 ± 0.005	0.002 ± 0.001
6h	Silymarin	0.665 ± 0.012	0.112 ± 0.003
	Taxifolin	0.034 ± 0.005	0.004 ± 0.001

Table 4. Quantity (mg) of silymarin and taxifolin permeated in PAMPA test.

Data are displayed as mean $\pm$ SD; n = 3.

This result agrees with the fact that the NE ameliorates the solubility of flavonolignans and much less that of taxifolin, probably due to different polarity and oil solubility of the constituents. In this case, the volume of acceptor compartment was 250 μ L and taxifolin is more easily quantifiable in this low volume respect to the dissolution test, and the effective permeability (P_e) values confirmed the increase of permeation of the extract respect to the aqueous solution.

The P_e of silymarin and taxifolin were calculated as reported in the literature (Chen et al., 2008; Hiremath et al., 2009). P_e was $93.5 \pm 1.2 \times 10^{-6}$ cm/sec for silymarin and $61.6 \pm 4.8 \times 10^{-6}$ cm/sec for taxifolin. In the case of aqueous solution P_e were $44.7 \pm 1.8 \times 10^{-6}$ cm/sec for silymarin and $21.0 \pm 3.7 \times 10^{-6}$ cm/sec for taxifolin. A recovery >80% required for an acceptable *in vitro* prediction was obtained. The values (data displayed as mean $\pm$ SD; n = 3) confirmed that NE also increased the permeability of the extract compared to the aqueous solution.

Caco-2 experiments

Caco-2 cells, human intestinal epithelial cell line, were used as a model to study drug absorption across the intestinal epithelium. Caco-2 cells are most widely and successfully used permeation models, which are employed to study passive diffusion, active transport, paracellular permeability and active efflux (Balimane et al., 2006).

The potential cytotoxicity of SM-loaded NE for Caco-2 cells was tested to find the highest non/low toxic concentrations to be used in transport experiments. After exposure for 4 h to a solution of NE diluted 40 times, the percentage of cell death was negligible compared to control (untreated cells), so this dilution was considered acceptable for transport experiments.

In the case of the aqueous solution, silymarin and taxifolin were not detected into the basolateral chamber (Table 5). On the contrary, in the case of NE these molecules penetrated into the basolateral chamber ($A \rightarrow B$), in particular 61.2% (4.63 mg) for silymarin and 79.5% (0.27 mg) for taxifolin, after 4 h of incubation. P_{app} values were reported in Table 5. A recovery >80% was obtained. Therefore, the formulations provided enhanced intestinal permeability of SM extract. This fact is undoubtedly due to the presence of surfactant used as stabilizer of the internal phase. As previously reported, the surfactants enhance drug permeability in many ways such as by increasing transcellular permeability and by inhibiting the efflux transport systems (Lin et al., 2007; Seljak et al., 2014). Labrasol and Labrafil are non-aqueous excipients used as solvents or co-solvents for absorption enhancing of oral and/or topical drug formulations (Bergamante et al., 2007; Hamid et al., 2009). They contain mono-, di- and triglycerides with mono- and di-esters of polyethylene glycol and fatty acids and they are applied as solubilizers, surfactants and absorption promoters (Kommuru et al., 2001). Labrasol showed high tolerance and low toxicity in rats (Hu et al., 2001). According to results of Delongea et al., 2010, 5 mL/kg/day was an acceptable and non-toxic volume for the use of Labrasol/Labrafil/Transcutol mixture as a vehicle for poorly water-soluble drugs. Furthermore, Labrasol, and to a lesser extend Cremophor, was shown to open tight junctions (Sha et al., 2005). The safety of components selected for our NE is another crucial aspect for oral administration.

To elucidate the possible mechanism involved in the penetration of Caco-2 monolayer, other studies were carried out by evaluating the permeability in the opposite direction ($B \rightarrow A$) and by using Verapamil as P-gp inhibitor, to obtain an indication of the involvement of an active process (Makhey et al., 1998).

Results are reported in Table 5. Efflux ratio resulted 0.82 for silymarin and 1 for taxifolin. No difference in permeation was observed and then the transport resulted mainly passive (Hubatsch et al., 2007).

The Caco-2 results confirmed the ability of NE to increase the permeation of the extract, as follows from PAMPA test.

In vitro transport studies by using PAMPA and Caco-2 models provided useful information about dissolution and permeation/absorption aspects, which further could be correlated to *in vivo* bioavailability studies. They proved that NE successfully enhances the permeation of extract compared to aqueous solution, by suggesting that the developed formulation will increase therapeutic effects of *Silybum marianum* extract.

Sample	Silymarin	Taxifolin
Extract (A→B)	n.d.	n.d.
NE (A→B)	51.50 × 10 ⁻⁶	66.93 × 10 ⁻⁶
NE (A→B) with verapamile	53.50 × 10 ⁻⁶	65.92 × 10 ⁻⁶
NE (B→A)	42.19 × 10 ⁻⁶	61.36 × 10 ⁻⁶

Table 5. P_{app} values (cm/s) of silymarin and taxifolin in Caco-2 test, after 4h;

A→B: from apical to basolateral compartment;

B→A from basolateral to apical compartment.

Data are displayed as mean ± SD; n = 3).

CONCLUSIONS

The developed NE represents a successful attempt to incorporate until 4% w/v of *Silybum marianum* commercial extract and to ameliorate its solubility significantly. It was suitable for oral administration concerning physical characteristics and safety of constituents. The system showed excellent chemical and physical stability during storage and the results from *in vitro* digestion assay clearly indicated the stability of the formulation in simulated gastrointestinal medium. *In vitro* transport studies by using PAMPA and Caco-2 models revealed that NE successfully enhances the permeation of extract compared to aqueous solution, suggesting that the developed formulation will increase therapeutic effects of *Silybum marianum* extract.

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Caco-2 cells studies were performed in collaboration with Dr. Cristina Luceri and Dr. Elisabetta Bigagli (NEUROFARBA, Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).

Original Paper

Thieme

Prediction of Permeation and Cellular Transport of *Silybum marianum* Extract Formulated in a Nanoemulsion by Using PAMPA and Caco-2 Cell Models*

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ENHANCED SOLUBILITY AND PERMEABILITY OF SALICIS CORTEX EXTRACT BY FORMULATING AS A MICROEMULSION

INTRODUCTION

Salicis cortex (SC), also known as willow bark, consists of the whole or fragmented dried bark of young branches or whole dried pieces of current year twigs of various species of the genus *Salix* including *S. purpurea* L., *S. daphnoides* Vill. and *S. fragilis* L. (Salicaceae). The main constituents are salicylic derivatives such as salicin and its related compounds, principally salicortin, 2'-O-acetylsalicortin and tremulacin. Other molecules include flavonoids, in particular the flavanones eriodictyol-7-O-glucoside, (+)- and (-)-naringenin-5-O-glucoside and naringenin-7-O-glucoside, catechin and polyphenols (ESCOP monographs, 1997). SC extract resulted beneficial in low back pain and in mild osteoarthritic and rheumatic complaints. The Commission E of the German Federal Health Agency has approved the use of willow bark preparations for the treatment of "diseases accompanied by fever, rheumatic ailments and headaches", in a daily dose equivalent to 60-120 mg salicin (Mills et al., 1996; Chrubasik et al., 2000; Chrubasik et al., 2001; Long et al., 2001). However, bioavailability of salicin after oral administration of willow bark extracts is low. Flavonoids also contribute to the overall therapeutic effect of the extract, but many studies have shown that poor solubility, fast metabolism and inadequate bioavailability hinder their clinical use (Thilakarathna & Rupasinghe, 2013).

In the present work an oil in water (O/W) microemulsion (ME) was developed to improve the solubility of SC extract, knowing that the solubility is a crucial factor for oral bioavailability. Microemulsions (MEs) are defined as transparent, thermodynamically stable mixtures of oil and water stabilized by one or more emulsifiers (Callender et al., 2017). Despite to the terminology, MEs consist of the smallest droplet sizes found in emulsion systems, specifically, ME droplet sizes range from 10 to 100 nm (McClements, 2012). Generally, MEs form spontaneously without the input of energy and require high amounts of surfactants or emulsifiers (Rosen & Kunjappu, 2012). MEs also possess unique characteristics as drug delivery systems, in particular they offer the possibility to administer drugs in liquid form and consequently increase their absorption rate avoiding disintegration processes and gastrointestinal enzymatic degradation (Shen et al., 2011). In addition, the small droplet sizes increase the surface area to volume ratio, key factor for drug absorption, and enhance the stability of the system. Furthermore, MEs can incorporate both hydrosoluble and lipophilic molecules frequently present in phytoterapeutics and in herbal extracts due to presence of a hydrophilic and a hydrophobic phase (Bilia et al., 2014; Bergonzi et al., 2014; Liu et al., 2016; Bilia et al., 2017). Finally, the ability of MEs to form spontaneously results in an economical formation process and they are suitable for almost any route of administration (Callender et al., 2017).

In previous studies salicin was incorporated into ME for topical application (Schonrock U, Steckel F, Kux U, Inoue K. U.S. Patent No. 5,876,737.1999; Washington, DC: U.S. Patent and Trademark Office) and willow bark extract was formulated into nanostructured lipid carriers to increase its antioxidant activity (Mitrea et al., 2015).

The present work represents the first attempt to increase the solubility of SC extract and its absorption after oral administration by formulating as ME.

In this study, an HPLC-DAD analytical method was optimized and validated for qualitative and quantitative characterization of SC extract. The selected components of ME were: triacetin as oil phase, Tween 20 as surfactant and Labrasol as co-surfactant. Empty and extract-loaded ME were physically and chemically investigated by Light Scattering techniques, electron microscopy and chromatographic analyses. Chemical and physical stability were also assessed during storage for one month and after incubation in simulated gastrointestinal conditions.

The ability of ME to increase the permeability of SC extract was evaluated *in vitro* by artificial membranes and Caco-2 cells, after preliminary cytotoxicity studies. The data obtained from a passive diffusion permeability assay and a cell-based test were compared to elucidate the permeation/absorption mechanism of salicylic derivatives and flavanones.

MATERIALS

Salicis cortex (willow bark) dry extract was supplied by Bionorica SE, Neumarkt, Germany. Sample number: PM 14-168, BNO 1455, Batch number: 0000083735, DEV: 8-17:1. The extract was standardized according to the Ph. Eur. criteria (minimum 5.0 per cent of total salicylic derivatives, expressed as salicin). Storage: tightly closed, dry, protected from light, 15-25°C.

Salicin (purity $\geq 99\%$, HPLC), naringenin (purity $\geq 99\%$, HPLC), naringenin-7-O-glucoside (purity $\geq 99\%$, HPLC) and eriodictyol-7-O-glucoside (purity $\geq 99\%$, HPLC) were from Extrasynthese (Genay Cedex, France), Tremulacin (purity $\geq 82\%$, HPLC) was from Sigma-Aldrich (Milan, Italy). Olive oil was from Olivia (Badia a Settimo, Florence, Italy); Wheat germ oil was purchased from Aboca (Sansepolcro, Arezzo, Italy); Sunflower oil was from Coop (Massarosa, Lucca, Italy); Tween 20 (Polyoxyethylene (20) sorbitan monolaurate), Tween 40 (Polyoxyethylene sorbitan monopalmitate), Cremophor EL (Castor Oil Fatty Acids, Ethoxylated Glycerol Esters), DL- α -Tocopherol acetate (Vitamin E), Triacetin, PEG 400, were purchased from Sigma-Aldrich (Milan, Italy); Hempseed oil, Almond oil, Borage oil, Propylene glycol were from Galeno srl (Comeana, Prato, Italy); Oleic acid was purchased from Carlo Erba Spa (Milan, Italy); Labrafil (2-[2,3-bis(2-hydroxyethoxy)propoxy]ethanol; hexadecanoic acid; octadecanoic acid), Labrasol ECH (Caprylocaproyl polyoxyl-8 glycerides) were a gift of Gattefossé (Saint Priest, France).

Ethanol analytical reagent, Acetonitrile and Methanol HPLC grade, Formic acid (98%), Cholesterol, Lecithin, Dichloromethane, DMSO, 1,7-octadiene (98%), Phosphate Buffered Saline (PBS) bioperformance certified, Lipase from porcine pancreas, Pepsin from porcine gastric mucose, Bile salts, HCl and NaOH were purchased from Sigma-Aldrich (Milan, Italy). All the substances for electron microscopy were bought from Electron Microscopy Sciences (Hatfield, USA). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA).

METHODS

Preparation of SC extract sample solution

Twenty millilitres of methanol were added to 500 mg of SC extract. The mixture was sonicated for 30 min in an ultrasonic bath, filtered and dried under vacuum. The obtained residue, precisely weighted, was solubilized in methanol for HPLC-DAD-TOF/MS studies.

HPLC-DAD and HPLC-TOF/MS settings

Qualitative and quantitative analyses were performed using an HP 1100 Liquid Chromatograph with a diode-array-detector (DAD) and controlled with an HP 9000 workstation (Agilent Technologies, Santa Clara, CA, USA). A 150 mm x 4.6 mm i.d., 5 μ m Zorbax Eclipse XDB, RP18 column was used (Agilent Technologies, Santa Clara, CA, USA). The eluents were: (A) formic acid/water pH 3.2 and (B) acetonitrile; the multi-step linear solvent gradient applied was: 0-5 min 15% B; 5-10 min 15-20% B; 10-15 min 20-30% B; 15-20 min 30-40% B; 20-25 min 40-15% B; equilibration time 10 min; oven temperature 30°C; flow rate 0.6 mL/min. The chromatograms were registered at different wavelengths: 210, 260, 280 and 350 nm.

HPLC-MS analyses were conducted by the same liquid chromatograph apparatus with a DAD coupled to TOF Mass Spectrometer equipped with an electrospray interface (all from Agilent Technologies, Santa Clara, CA, USA). Analysis parameters were set using a negative ion mode with spectra acquired over a mass range of 100-1000 m/z. The conditions of electrospray source were as follows: drying gas (N₂) temperature, 350°C; drying gas flow rate, 6 L/min; nebulizer 20 psi; capillary voltage, 4000 V; fragmentation, 150 V; skimmer, 60 V. The other parameters were the same of HPLC-DAD analyses.

Method validation

HPLC-DAD method validation was performed according to international regulatory guidelines (ICH, 1996; EMA, 2012). Linearity, accuracy, precision, reproducibility, repeatability, limit of quantification (LOQ) and limit of detection (LOD) were estimated using salicin and naringenin-7-*O*-glucoside reference standards as representative compounds of salicylic derivatives and flavanones, respectively. Linearity was investigated after three injections of each level of five different concentrations. The regression parameters were calculated by linear regression analysis.

Accuracy was determined by evaluating the percentage recovery of salicin and naringenin-7-*O*-glucoside from three preparations of SC extract at different concentrations (2.00, 2.67 and 4.00 mg/mL). Each sample was injected three times. 100 µg/mL of salicin and 50 µg/mL of naringenin-7-*O*-glucoside were added separately to the three batches of the extract.

The reproducibility was calculated after ten injections of salicin (0.65 mg/mL) and naringenin-7-*O*-glucoside (0.50 mg/mL) methanolic solutions.

The repeatability was evaluated estimating the relative standard deviation of salicylic derivatives and flavanones content after three injections of each SC extract solutions at three different concentrations (2.04, 3.30 and 4.03 mg/mL).

The intermediate precision was determined injecting six times on three different days the samples previously used for the repeatability evaluation.

The peak purity of salicin and naringenin-7-*O*-glucoside was investigated overlaying the UV-spectra at the beginning, at the apex and at the end of the peaks. Limit of detection and limit of quantification of salicin and naringenin-7-*O*-glucoside were estimated by means of the signal-to-noise ratio. A signal-to-noise ratio 3:1 is generally considered acceptable for estimating the detection limit. The sample that produces a signal-to-noise ratio of approximately 10:1 corresponds to the concentration at which the analyte can be reliably quantified.

Selection of oil and surfactants

Solubility studies of SC extract were performed in various oils (olive oil, wheat germ oil, sunflower oil, almond oil, hemp oil, borage oil, triacetin, oleic acid and tocopherol acetate) and surfactants (Tween 20, Cremophor EL, Labrasol, peg 400 and ethanol) to select the best components for ME formulation. Increased amounts of SC extract were added to an exact volume of each selected vehicle until saturation and the resulted samples were stirred for 24 h at room temperature. Then the mixtures were centrifuged at $13148 \times g$ for 10 min and the concentration of salicylic derivatives and flavanones was determined using the chromatographic method reported in the previous section, after appropriate dilution with methanol/dichloromethane (3:2 volume ratio) mixture.

Development of pseudoternary phase diagram

Based on solubility studies a pseudoternary phase diagram was constructed by water titration technique using triacetin as the oil phase, Labrasol as surfactant and Tween 20 as co-surfactant. Labrasol and Tween 20 were mixed at different ratios under vigorous stirring to obtain the surfactant-cosurfactant mixture (S_{mix}). Then various combinations (from 1:9 to 9:1 volume ratio) of triacetin and S_{mix} were tested, and water was added dropwise to each blend, under stirring at room temperature. During the addition of water, the changes in the samples feature were monitored to determinate if emulsion, gel or ME was present. The points from transparent to cloudy and cloudy to transparent were considered as emulsion and ME, respectively.

Solubility of SC extract into microemulsion

After the elaboration of the pseudoternary phase diagram, the maximum loading content of SC extract into the formulation was evaluated adding an increasing amount of extract powder to the ME under stirring. The samples were shaken for 24 h at RT, sheltered from the light. Afterward, the undissolved powder was removed by centrifugation at $13148 \times g$ for 10 min and salicylic derivatives and flavanones content was quantified by chromatographic analyses after appropriate dilution with $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (3:2 v/v) mixture.

Droplet size, homogeneity and ζ -potential measurements

The Z_{average} , the particle size distribution expressed as polydispersity index (PDI) and ζ -potential were analyzed by a Light Scattering apparatus Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK). All the measurements were performed in triplicate after proper dilutions with distilled water.

Morphological examination

Morphological features of the formulations were examined by TEM JEOL 1010 (Tokyo, Japan). PTA 1% w/v was used as negative stain for empty and SC-ME.

Chemical and physical stability

Empty and SC-ME were stored in sealed glass containers at 4°C and 25°C for 30 days, to estimate the shelf life of the optimized formulations. Chemical and physical properties were checked periodically monitoring transparency, phase separation, colour variation as the changes in particle size, ζ -potential and extract concentration by visual observations, Light Scattering and HPLC analyses.

Stability in simulated gastrointestinal fluids

Stability of developed ME in simulated gastrointestinal fluids was evaluated by adding an equal volume of simulated gastric fluid (SGF). The composition of SGF was: 0.32% w/v pepsin, 2 g of NaCl and 7 mL HCl dissolved in 1 L water (pH adjusted to 2.0 using 1M HCl). The resulted mixture was kept at 37°C under continuous shaking for 2 hours, after which droplet size, PdI and SC extract content were measured (Aditya et al., 2013).

After digestion in SGF, the sample was incubated in simulated intestinal fluid (SIF) containing digestive enzymes (lipase 0.4 mg/mL, bile salts 0.7 mg/mL, pancreatin 0.5 mg/mL and CaCl₂ 750 mM at pH 7.0) for 2 h at 37°C. At the end of the experiment physical and chemical stability were investigated by DLS and HPLC analyses (Aditya et al., 2013).

In vitro Parallel Artificial Membrane Permeability Assay (PAMPA)

The experiments were performed using the optimized conditions previously reported with slight modifications (Bergonzi et al., 2014). Lecithin and cholesterol mixture, 10 g/L and 8 g/L, respectively, previously dissolved in 1,7-octadiene was applied to each PVDF filter as the artificial membrane. DMSO/PBS (0.05 mL/mL, pH 7.4) combination was chosen as acceptor buffer. Immediately after the deposition of the artificial membrane (5 µL), 250 µL of the extract containing samples (saturated aqueous solution and SC-ME) were added into the donor compartments. After 4 hours of incubation flavanones and salicylic derivatives content into donor and receptor compartment was evaluated by HPLC.

Compounds' permeability and recovery were calculated according to the equation reported in the literature (Wohnsland & Faller, 2001; Sugano et al., 2001):

$$P_e = C \times -\ln \left(1 - \frac{[\text{Compound}]_A}{[\text{Compound}]_{eq}} \right); \text{ where } C = \frac{V_D \times V_A}{(V_D + V_A) \times A \times t};$$

$$[\text{Compound}]_{eq} = \frac{[\text{Compound}]_D \times V_D + [\text{Compound}]_A \times V_A}{(V_D + V_A)}$$

$$\text{Recovery (\%)} = \frac{[\text{Compound}]_D \times V_D + [\text{Compound}]_A \times V_A}{([\text{Compound}]_{D_0} \times V_D)} \times 100$$

P_e = effective permeability (cm/s). A = effective filter area (cm²), V_D and V_A = volumes in the acceptor and the donor compartments (mL), t = incubation time (s), $[\text{Compound}]_A$ and $[\text{Compound}]_D$ = concentrations of the compound in the acceptor and the donor compartments at time t , $[\text{Compound}]_{D_0}$ = compound concentration in the donor compartment at time 0.

Caco-2 cell culture and cell viability studies

The human colon carcinoma Caco-2 cell line was kindly provided by Professor Emanuela Masini (University of Florence, Italy). Caco-2 cells were seeded in DMEM (or DME) supplemented with 20% heat-inactivated fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin, grown at 37°C in a humidified atmosphere containing 5% CO₂ and passaged at a confluence of 80%.

To assess cell viability after samples exposure, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was performed. Cells were transferred to flat bottom 96-well tissue culture plates at a seeding density of 5x10³ cells/well and allowed to grow for 24 h. When the cells were approximately 80% confluent, they were incubated with different concentrations of SC-ME for 24 h.

Then the cells were exposed to MTS solution and allowed to incubate for 24 h at 37°C. The product of the reaction was measured at 490 nm using a spectrophotometer. Cell death was expressed as a percentage compared to the values obtained from the positive controls, i.e. cells incubated only in DMEM.

Caco-2 transport studies

For permeability assays, cells were seeded at a density of 50×10^3 cells/well in cell culture inserts with polyethylene terephthalate (PET) membranes (0.4 μm pore size, 1.12 cm^2 growth surface area; BRAND). DMEM was added into the apical (A) and basolateral (B) compartments and daily changed. After seeding, Caco-2 cells were let to differentiate for three weeks.

Lucifer yellow (LY) ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$) at a concentration of 100 mM was used as integrity control marker (Iacomino et al., 2013). After dilution in transport medium (HBSS with Ca^{2+} , Mg^{2+} , 25mM HEPES, pH 7.4), LY was put into the A and after 1 h of incubation the amount permeated into the B chamber was calculated using a fluorescence plate reader to estimate the percentage of cleavage. The maximum permeability of LY to prove the integrity of the layers was established to be not more than 3% respect to the initial amount added into the AP compartment.

Before the transport study, DMEM was replaced with preheated (37°C) transport medium. After the cell monolayer was equilibrated for 30 min at 37°C, Caco-2 cells were exposed to 0.5 mL SC-ME diluted in the buffer medium and SC aqueous solution, while the B chamber contained only HBSS. At the end of the assay the concentration of salicylic derivatives and flavanones was calculated both in A and B chamber by HPLC and expressed as salicin and naringenin 7-O-glucoside, respectively, as reported in the method validation section. Furthermore, the integrity of the layer was re-evaluated with the LY permeability assay as described above.

Apparent permeability coefficients (P_{app} , cm/s) and recovery for active constituents of SC extract were calculated according to the equation reported in the literature (Hubatsch et al., 2007; Palmgren et al., 2006; Heikkinen et al., 2009):

$$P_{app} = \frac{\Delta Q / \Delta t}{C_0 \times S}$$

where $\Delta Q / \Delta t$ indicates linear appearance rate of mass in the B; C_0 , initial concentration in A; and S, surface area (i.e., 1.12 cm²). Recovery was calculated as reported in PAMPA section.

Statistical analysis

Experiments were repeated three times and results expressed as a mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

HPLC-DAD and HPLC-TOF/MS analyses

The first step of this study concerned the qualitative and quantitative characterization of SC commercial extract by HPLC-DAD analytical method. The wavelengths of 210, 260, 280 and 350 nm were selected for acquiring chromatograms of salicylic derivatives and flavanones. In order to achieve a good resolution, various percentages of mobile phase mixtures at different flows and several RP-C18 columns were tested. Finally, the conditions reported in the experimental section were selected because the peaks related to the characteristics constituents of the extract resulted separated. The chromatogram of sample solution selecting a wavelength of 280 nm with identified compounds (salicin, salicortin, eriodictyol-7-*O*-glucoside, (+)- and (-)-naringenin-5-*O*-glucoside, naringenin-7-*O*-glucoside, isosalipurposide, tremulacin, naringenin) is reported in Figure 1.

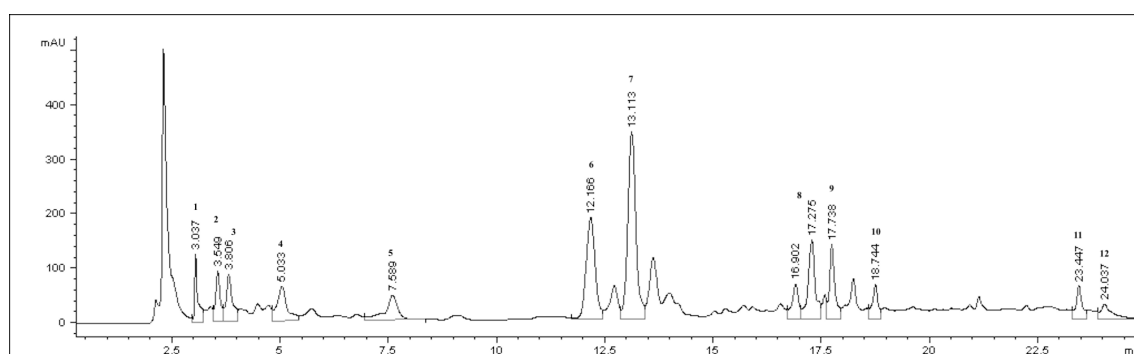


Figure 1. HPLC/DAD chromatogram of SC extract at 280 nm.

1. Salicin; 2. Salicin derivative; 3. Salicin derivative; 4. Salicin derivative; 5. Salicin derivative;
6. Salicortin; 7. Eriodictyol-7-*O*-glucoside; 8. (+)- and (-)-Naringenin-5-*O*-glucoside;
9. Naringenin-7-*O*-glucoside; 10. Isosalipurposide; 11. Tremulacin; 12. Naringenin.

The identification of SC extract constituents was obtained comparing UV spectrum, retention time and MS data with those of standards or with the literature data. The structures and UV/MS data of (+)- and (-)-naringenin-5-*O*-glucoside, naringenin-7-*O*-glucoside were reported in the Table 1 (Kammerer et al., 2005).

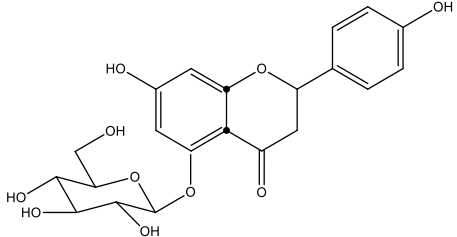
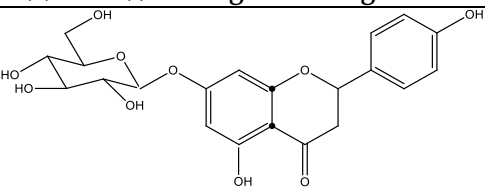
Compound	Retention Time (min)	λ max (nm)	m/z	MS fragment (m/z)
 (+)- and (-)-Naringenin-5-<i>O</i>-glucoside	16.90 17.27	280	434.3	479.3; 433.4; 271.1
 Naringenin-7-<i>O</i>-glucoside	17.73	280	434.3	479.3; 433.4; 271.1

Table 1. Retention time, UV data and MS fragmentors of two naringenin derivatives.

The HPLC-DAD analytical method was validated according to the criteria established by ICH guidelines (ICH, 1996; EMA Guidelines, 2012) relatively to salicin and naringenin-7-*O*-glucoside, the most representative compounds of salicylic derivatives and flavanones, respectively.

Firstly, linearity range of response was determined on five levels of concentration of salicin and naringenin-7-*O*-glucoside with three injections for each level. Salicin and naringenin-7-*O*-glucoside showed a linear response from 100 to 650 µg/mL with a coefficient of linear correlation ≥ 0.993 .

About the reproducibility, the relative standard deviation for salicin and naringenin-7-*O*-glucoside solutions resulted 0.81% and 0.55%, respectively.

The repeatability of the method was evaluated using methanolic solutions of SC extract at three different level of concentration. The relative standard deviation (R.S.D.) resulted 1.86%, 3.09% and 2.08% for salicylic derivatives and 1.80%, 2.42% and 2.19% for flavanones.

Intermediate precision relative to salicin and naringenin-7-*O*-glucoside was also calculated. For first sample (2.04 mg/mL) R.S.D. of salicin was 1.90%, 2.86%, and 1.90%; for naringenin-7-*O*-glucoside R.S.D. resulted 1.82%, 2.14%, and 2.03%. Precision interday for salicin was 2.25% and for naringenin-7-*O*-glucoside was 1.99%. For second sample (3.30 mg/mL) R.S.D. of salicin was 3.10%, 1.95%, and 2.03%; for naringenin-7-*O*-glucoside was 2.14%, 1.41%, and 0.93%. The precision interday for salicin was 2.36% and for naringenin-7-*O*-glucoside was 1.49%. For the third sample (4.03 mg/mL) the R.S.D. of salicin was 1.11%, 0.73%, 0.72% and 0.96%, 1.42% and 1.44% for naringenin-7-*O*-glucoside. The precision interday for salicin was 0.85% and for naringenin-7-*O*-glucoside was 1.27%.

The accuracy was determined by analyzing the percentage recovery of salicin and naringenin-7-*O*-glucoside in the solutions of SC extracts in three concentration levels (2.00, 2.67 and 4.00 mg/mL) after spiking 100 µg/mL of salicin and 50 µg/mL of naringenin-7-*O*-glucoside separately to the extract solutions. The percentages recoveries of salicin were $106.67 \pm 8.92\%$, $103.99 \pm 1.32\%$, and $106.84 \pm 1.45\%$ and for naringenin-7-*O*-glucoside were $119.70 \pm 0.42\%$, $123.56 \pm 1.11\%$ and $111.00 \pm 0.25\%$.

About the peak purity (purity factors were 999.996 for salicin and 999.999 for naringenin-7-*O*-glucoside), no deviations were seen overlaying the UV-spectra at the beginning, at the apex and at the end of salicin and naringenin-7-*O*-glucoside peaks.

The limits of detection of salicin and naringenin-7-*O*-glucoside resulted 3.25 ng and 0.25 ng respectively (the signal-to-noise ratio was 3), while the limits of quantification (signal-to-noise ratio = 10) of these molecules resulted 16.25 ng and 2.51 ng, respectively.

Thus, based on obtained results, HPLC-DAD conditions could be considered specific and useful for the characterization of SC extract. The percentage w/w of each compound in SC extract was: Salicin (1) 0.8%; Salicin derivative (2) 0.6%; Salicin derivative (3) 0.7%; Salicin derivative (4) 0.9%; Salicin derivative (5) 1.2%; Salicortin (6) 4.3%; Eriodictyol-7-*O*-glucoside (7) 3.0%; (+)- and (-)-Naringenin-5-*O*-glucoside (8) 1.1%; Naringenin-7-*O*-glucoside (9) 0.5%; Isosalipurposide (10) 0.5%; Tremulacin (11) 0.5%; Naringenin (12) 0.1%. Total flavanones and salicylic derivatives content resulted 4.7% and 9.5%, respectively.

Selection of oil and surfactants

The components of the ME were selected by the solubility study of SC extract in various oils and surfactants (Table 2). Selected oils were olive oil, wheat germ oil, sunflower oil, almond oil, hemp oil, borage oil, triacetin, and oleic acid and tocopherol acetate. Surfactants were Tween 20, Cremophor EL, Labrasol, PEG 400 and ethanol.

Oils	Salicylic derivatives	Flavanones
	(mg/mL)	(mg/mL)
Triacetin	3.52 ± 0.67	0.93 ± 0.11
Olive oil	-	-
Sunflower oil	-	-
Hemp oil	-	-
Almond oil	-	-
Wheat germ oil	-	-
Borage oil	-	-
Oleic acid	-	-
Tocopherol acetate	-	-
Surfactants		
Tween 20	4.70 ± 1.31	1.21 ± 0.01
Cremophor EL	3.14 ± 0.11	1.02 ± 0.01
Labrasol	5.40 ± 1.90	1.64 ± 0.71
PEG 400	5.50 ± 1.70	1.82 ± 0.25
Ethanol	4.71 ± 0.18	1.82 ± 0.28

Table 2. Solubility of SC extract constituents in different vehicles.

Results are expressed as means ± standard deviation; n = 3

Safety of the vehicles and solubility of the extract are the most critical factors in choosing oil and surfactants. Among the oils tested, the solubility of SC extract was highest in triacetin, selected as the oil phase. Triacetin, or glyceryl triacetate, is widely present in oral pharmaceutical formulations because non-toxic, non-irritating and biocompatible. Moreover, its water solubility and mixability make it an ideal oil phase for ME's development (Fiume & Panel, 2003; Sharma et al., 2010).

Regarding the selection of surfactants, it was demonstrated that the hydrophilic-lipophilic balance value appropriate to form a stable o/w ME must be greater than 10 (Shinde et al., 2018). For this reason, Tween 20 and Labrasol were chosen as surfactant and co-surfactant, respectively, also considering their high solubilizing capacity toward the SC extract. Tween 20 (also known as polysorbate 20) is a non-ionic surfactant, used to increase oral bioavailability of drugs due to its capacity to improve solubility and inhibit ABC-transporter mediated cellular efflux (Woodcock et al., 1992; Nielsen et al., 2016). Labrasol consists of a small fraction of mono-, di- and triglycerides and mainly PEG-8 (MW 400) mono- and diesters of caprylic and capric acid; in this research it was used together with Tween 20 to achieve transient negative interfacial tension and fluid interfacial film. It is also reported that Labrasol can increase membrane permeability and inhibit secretory systems in the intestinal epithelium (Sha et al., 2005), thus it could be useful to increase oral absorption of SC extract.

Development of pseudoternary phase diagram

Water was added dropwise to oil/surfactant-cosurfactant (S_{mix}) mixture to obtain the existence region of ME. Ternary compositions were visually analyzed to determine if transparent ME, gel, a cloudy or a milky emulsion was formed. Figure 2 depicts the phase diagram constructed using different combinations of oil, surfactants and water.

Final composition of developed ME was 2.5 g of triacetin (20% w/w) chosen as the oil phase, 2.5 g of Tween 20 (20% w/w) as surfactant, 2.5 of Labrasol (20% w/w) as co-surfactant and 5 mL of deionized water (40% w/w). ME became light brown after the addition of the extract, but it resulted transparent, proof of the stability of the formulation.

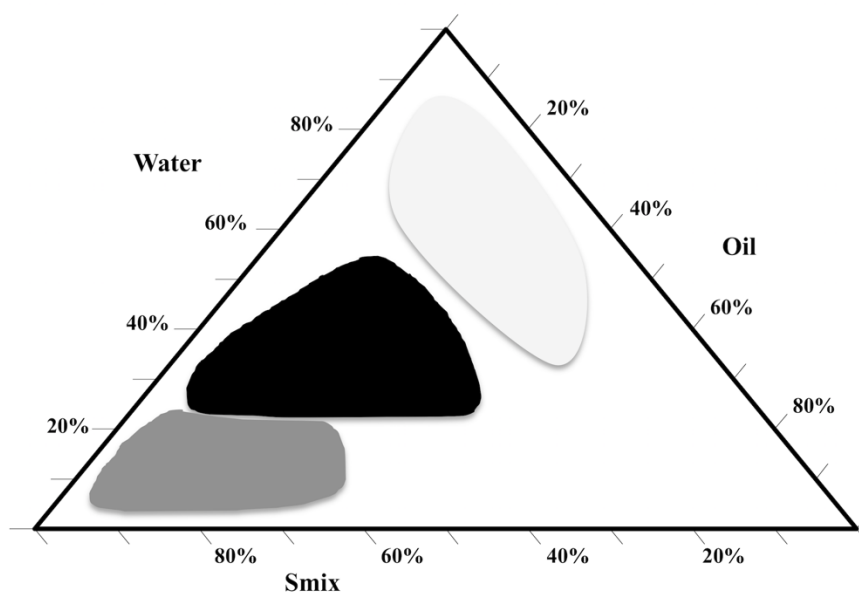


Figure 2. Pseudo-ternary phase diagram of microemulsion.
Black: microemulsion; grey: gel; light grey: turbidity

Solubility of SC extract into microemulsion

The solubility of SC extract into developed ME was evaluated by adding increasing amounts (from 5 mg/mL until 50 mg/mL) to the system. Resulting samples were visually checked and analyzed by HPLC-DAD after the elimination of undissolved extract.

No phase separation or turbidity was detected, and the SC extract resulted solubilized entirely into ME at the concentration of 40 mg/mL, while in water the amount of dissolved extract resulted below of 7 mg/mL.

Furthermore, as showed in Table 3, ME increased considerably the solubility of salicylic derivatives and flavanones respect to water (about 3.6 times for salicylic derivatives and about 2 times for flavanones). This result is probably due to the synergistic effect between triacetin, water and S_{mix} and to chemical interactions between ME ingredients and extract's compounds.

SC extract-loaded microemulsion	Salicylic derivatives (mg/mL)	Flavanones (mg/mL)
5 mg/mL of extract	2.01 ± 0.10	0.75 ± 0.05
10 mg/mL of extract	3.82 ± 0.24	1.31 ± 0.08
20 mg/mL of extract	7.35 ± 0.18	2.40 ± 0.01
40 mg/mL of extract	16.00 ± 1.31	5.61 ± 0.12
SC extract in water (control)	4.40 ± 0.20	2.81 ± 0.01

Table 3. Solubility of Salicis cortex extract at different concentrations in ME compared to water. Results are expressed as means ± standard deviation; n = 3

Characterization of ME

DLS analysis revealed that empty ME was composed by dispersed droplets with average diameter around 30-40 nm and PDI below 0.2 and confirmed that in the presence of 40 mg/mL of SC extract, size and homogeneity of the system did not change significantly. ζ -potential values ranged from -11 to -14 mV, indicating that developed ME were negatively charged and signifying the stability of the formulations (Table 4). Indeed, it is known that an increase of electrostatic repulsive forces between ME droplets prevent coalescence phenomena.

Sample	Size (nm)	PdI	ζ -potential (mV)
ME	28.60 ± 0.08	0.19 ± 0.01	-14.22 ± 0.20
SC-ME (40 mg/mL of extract)	35.81 ± 0.13	0.20 ± 0.01	-11.25 ± 0.13

Table 4. Physical characterization of microemulsion and SC extract-loaded microemulsion.

Results are expressed as means $\pm$ standard deviation; n = 3.

TEM observations proved the presence of spherical and non-aggregated droplets with size less 50 nm both for empty and SC-ME (Figure 3).

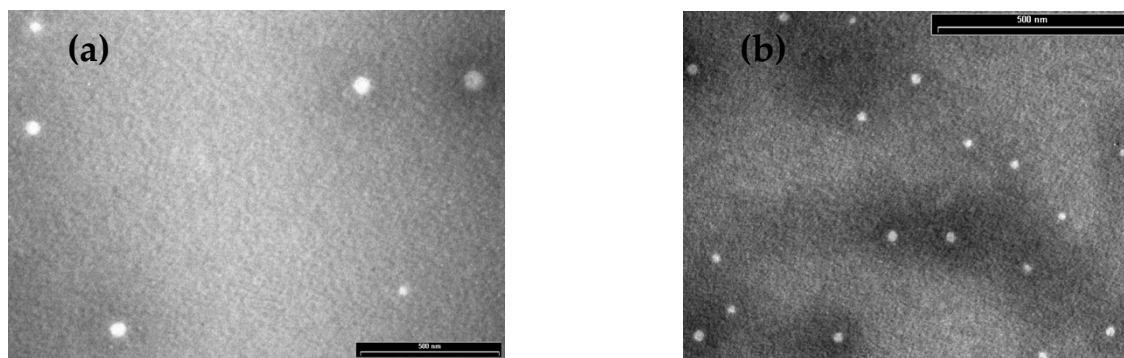


Figure 3. TEM images of empty microemulsion (a) and SC extract-loaded microemulsion (b).

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

According to the data reported in literature about physical and morphological properties of MEs (Zhang et al., 2008), these results suggest that the optimized formulation represents a suitable vehicle for oral delivery of SC extract.

Stability studies

Stability of empty and SC-ME was evaluated at 4°C and 25°C for four weeks. During this period, the systems were stable: no phase separation or creaming was observed. Moreover, physical parameters, i.e. size, homogeneity and ζ -potential values resulted unchanged. The concentration of salicylic derivatives and flavanones, calculated by HPLC analysis, was not changed at 4°C and 25°C. The obtained results suggested that the developed formulations were stable during the storage period and they were able to prevent the degradation of loaded molecules.

It is known that gastrointestinal enzymes will digest coarse emulsions, so one of the aims of this work was the development of innovative formulation able to protect SC extract from the unfavourable environment after oral administration. For this purpose, physical and chemical properties of ME were assessed after exposure to SGF and SIF. After 2 h of incubation in gastric conditions followed by 2 h of incubation in a simulated intestinal environment, size remained around 40 nm and PdI lower than 0.25 (Table 5).

Sample	Size (nm)	PdI	Salicylic derivatives	Flavanones
			(mg/mL)	(mg/mL)
SGF	36.11 ± 2.10	0.22 ± 0.05	16.01 ± 0.18	5.40 ± 0.05
SIF	36.71 ± 5.60	0.23 ± 0.06	16.10 ± 0.12	5.42 ± 0.08

Table 5. Physical and chemical stability of SC-ME in simulated gastrointestinal fluids.

Results are expressed as means ± standard deviation, n = 3.

Coalescence or separation phenomena were not observed after visual inspection. This stability in acidic pH and in intestinal conditions may be due to the presence of non-ionic surfactants, Tween 20 and Labrasol, which reduce the flocculation and coalescence phenomena.

The ME can prevent the enzymatic degradation of entrapped extract before intestinal absorption, in fact, the concentration of the main constituents of SC did not decrease during the time of the assay (Table 5). Furthermore, optimized ME demonstrated excellent physical stability in gastrointestinal conditions, thus it represents a promising strategy to deliver a high quantity of extract and to enhance its intestinal permeability when orally administered.

In vitro parallel artificial membrane permeability assay (PAMPA)

Parallel Artificial Membrane Permeability Assay (PAMPA) was performed to estimate passive transcellular permeability. PAMPA is a non-cell based permeability model but it is considered robust, reproducible and fast (Kansy et al., 1998; Alvarez-Figueroa et al., 2011; Nekkanti et al., 2016). It was demonstrated that PAMPA could be used to predict the passive permeability through the gastrointestinal tract of constituents in a complex plant extract (Petit et al., 2016). Based on the results obtained in previous studies (Bergonzi et al., 2014), this *in vitro* assay resulted useful not only in the preformulation studies of single molecules but also for herbal extracts loaded into innovative drug delivery systems.

Considering that most compounds reach the bloodstream by passive mechanisms, PAMPA has been proposed as a helpful complement to Caco-2 assay to predict the oral absorption of SC extract.

The effective permeability (P_e) values and the permeated amounts of salicylic derivatives and flavanones are reported in Table 6.

The maximum P_e values were found after 4 h of incubation. Furthermore, the amount of SC extract diffused into the acceptor compartment and P_e of salicylic derivatives and flavanones have considerably improved in the case of ME formulation respect to water solution. This result is may be due to the increased lipophilicity of the extract.

A recovery above 80% was achieved for SC-ME and for the aqueous solution (Table 6), as required for an acceptable permeation prediction (Palmgren et al., 2006; Hubatsch et al., 2007; Heikkinen et al., 2009). Moreover, this value means that there was neither relevant SC extract membrane retention nor binding to the surface of the filter.

The results confirm that developed formulation represents a successful attempt to ameliorate passive permeation of SC.

Sample	P_e Salicylic derivatives ($\times 10^{-6}$ cm/s)	P_e Flavanones ($\times 10^{-6}$ cm/s)	Salicylic derivatives permeated (μ g)	Flavanones permeated (μ g)	Recovery (%) for salicylic derivatives	Recovery (%) for flavanones
SC-ME (40 mg/mL)	42.0 ± 3.2	36.0 ± 5.4	380 ± 10	120 ± 30	91.0 ± 1.1	98.2 ± 1.5
SC extract (aqueous solution)	7.4 ± 0.6	3.6 ± 0.2	20 ± 1	7 ± 1	93.1 ± 0.5	83.1 ± 2.1

Table 6. Effective permeability coefficients (P_e) of salicylic derivatives and flavanones and quantity permeated (μ g) in PAMPA.

Results are expressed as means $\pm$ standard deviation, $n = 3$.

Caco-2 experiments

Permeation studies were also performed using a cell-based model in order to complete *in vitro* characterization of SC-ME. Indeed, an understanding of oral absorption mechanisms is crucial for the elaboration of a novel oral formulation. For this purpose, Caco-2 cells are considered the most predictive *in vitro* model to estimate not only passive intestinal diffusion, as previously described for PAMPA, but also active transport processes, paracellular permeability and active efflux (Palmgren et al., 2006; Hubatsch et al., 2007; Heikkinen et al., 2009). However, for the differentiation and the preparation of a fully functional monolayer, cells require around three weeks.

Firstly, the potential cytotoxicity of SC-ME was investigated to select the concentration for permeation studies. As reported in Figure 4, optimized formulation had very low toxicity proved by the cell viability value above that 80%. SC-ME was diluted 50-fold for permeation studies considering that after 24 h of incubation at this concentration, the percentage of cell death was negligible compared to the positive control. This is an encouraging result considering that ME undergoes a dilution from 1:200 to 1:1000 with biological fluids after oral administration.

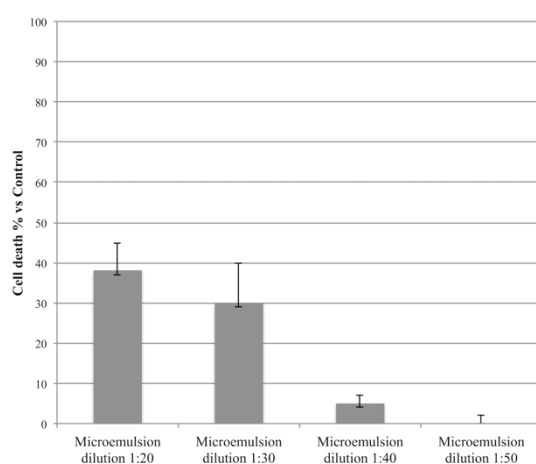


Figure 4. Caco-2 cell viability assessed by MTS assay after 24 h of exposition to diluted SC-ME. Results are expressed as means $\pm$ standard deviation of at least three experiments.

Furthermore, Lucifer Yellow (LY) was used to prove the absence of monolayer cleavage before performing the transport experiments. The LY passage resulted less than 2%, confirming the integrity of the layer.

P_{app} values are given in Table 7. It can be observed that the permeation of salicylic derivatives and flavanones was improved when SC extract is formulated into ME respect to the saturated aqueous solution. P_{app} values were found above than 1×10^{-5} signifying high permeability properties of ME.

Sample	P_{app} Salicylic derivatives ($\times 10^{-6}$ cm/s)	P_{app} Flavanones ($\times 10^{-6}$ cm/s)	Salicylic derivatives permeated (μ g)	Flavanones permeated (μ g)	Recovery (%) for salicylic derivatives	Recovery (%) for flavanones
SC-ME (40 mg/mL)	19.08 ± 0.21	16.01 ± 1.02	98.0 ± 1.1	29.1 ± 0.1	100 ± 0	99.02 ± 0.03
SC extract (aqueous solution)	2.14 ± 0.03	1.30 ± 0.01	3.0 ± 0.1	1.2 ± 0.2	100 ± 0	99.11 ± 0.02

Table 7. Apparent permeability coefficients (P_{app}) of salicylic derivatives and flavanones and quantity permeated (μ g) in Caco-2 assay.

Results are expressed as means $\pm$ standard deviation, $n = 3$.

This result is probably due to the small droplets of ME characterized by a big surface area, which leads to increased solubility and absorption. Moreover, non-ionic surfactants such as Labrasol and Tween 20, also contributed to increase extract solubility, avoid degradation phenomena, extend the contact time with the absorption site, increase endocytic and transcellular pathways and inhibit P-gp efflux activity (Woodcock et al., 1992; Sha et al., 2005; Nielsen et al., 2016).

As in the case of PAMPA, also in this case, the recovery resulted above than 80%, as required for acceptable *in vitro* permeation prediction (Table 7).

The obtained results clearly demonstrated that both *in vitro* models, i.e. PAMPA and Caco-2 cell line, could be synergistically applied for the correct estimation of drugs permeability and intestinal absorption and future studies will be aimed to evaluate pharmacokinetic parameters to confirm the potential of developed ME.

CONCLUSIONS

ME as a novel oral delivery system to enhance the solubility and intestinal absorption of Salicis cortex extract was developed. Triacetin was the oil phase, Tween 20 and Labrasol were surfactant and co-surfactant, respectively.

ME considerably increased SC extract solubility compared to water and based on obtained results concerning size, homogeneity and ζ -potential could be orally administered. SC-ME was stable during storage at different temperatures (25°C and 4°C) until four weeks. Furthermore, the formulation was also stable after incubation into simulated gastrointestinal environment. Transport experiments with PAMPA and Caco-2 model showed that the formulation was successful in enhancing the permeation of main constituents of SC extract. Thus, based on obtained results, developed ME could be used as alternative oral formulation and we expect that it will enhance the bioavailability and, finally, therapeutic effects of SC extract.

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Caco-2 cells studies were performed in collaboration with Dr. Cristina Luceri and Dr. Elisabetta Bigagli (NEUROFARBA, Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).

Original Papers

Thieme

Enhanced Solubility and Permeability of Salicis cortex Extract by Formulating as a Microemulsion*

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SOLID LIPID NANOPARTICLES AND CHITOSAN-COATED SOLID LIPID NANOPARTICLES AS PROMISING TOOL FOR SILYBIN DELIVERY: FORMULATION, CHARACTERIZATION, AND *IN VITRO* EVALUATION

INTRODUCTION

Lipid based-nanocarriers, such as micro and nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, represent a successful example of nanoformulations applied to overcome the limitations of natural compounds (Bilia et al., 2017; Puglia et al., 2017). They can be developed in small particle sizes ranging from micro to nanometers for increasing the absorption in the hydrophilic environment of the gastrointestinal tract.

Solid lipid nanoparticles (SLNs) have been proposed as biopolymer-based nanocarriers of therapeutic agents and diagnostics by many researchers. The advantages of these colloidal carriers as drug delivery system are: the possibility of controlled drug release and drug targeting, protection of compound against chemical degradation, no biotoxicity of the carrier, biodegradation and no problems concerning large-scale production (Muller et al., 1996; Bilia et al., 2017). One of the advantages of nanoparticles, when administered orally, is that they can be absorbed through different mechanisms. SLNs can enhance the transport of the encapsulated drug. This fact is related to different mechanisms such as mucoadhesion, nanoparticle internalization and permeation enhancing effect (Hussain et al., 2001; Bilia et al., 2014; Righeschi et al., 2016).

In this work, SLNs were proposed as nanocarrier to improve the solubility and the intestinal absorption of silybin (Sb). Sb is a flavonolignan, the major biologically active component in silymarin, a standardized extract derived from seeds and fruits of *Silybum marianum* L. Gaertn. It is widely used for the treatment of various acute and chronic liver toxicities, inflammation, fibrosis and oxidative stress. The compound has low toxicity and exerts significant anti-carcinogenic and anti-inflammatory effects (Saliou et al., 1998; Z. L. Zi & Agarwal, 1999). Many studies indicate that Sb is also active against different carcinomas and it has been very recently proposed to be beneficial in type 2 diabetes patients. Many articles demonstrated decreases in both fasting and mean daily glucose, triglycerides and total cholesterol levels (Velussi et al., 1997; Lirussi et al., 2002; Huseini et al., 2006).

However, Sb is a low water soluble and low permeable compound, belonging to biopharmaceutical class IV type that faces stipulated requirements in delivery design. Thus, to overcome these limitations, Sb was encapsulated into SLNs made of stearic acid and brij 78 (Sb-SLNs) and into chitosan-coated SLNs (Sb-CS-SLNs). Brij 78 was chosen as non-ionic surfactant to improve suspension stability. Furthermore, brij 78 confers stealth properties to nanoparticles (J. Q. Zhang et al., 2007), it can avoid degradation phenomena in the gastrointestinal environment and prevents the burst release of the entrapped compound (Kaur et al., 2008). Moreover, brij 78, accelerates colloid transport across the epithelium and provides reservoir function to nanoparticles carrying hydrophobic drugs (J. Q. Zhang et al., 2007).

Chitosan was used to improve the interaction of SLNs with the intestinal mucosa and consequently drug absorption. It is a polysaccharide derived by deacetylation of chitin and, in addition to being biocompatible and biodegradable material, exerts mucoadhesive properties, which can promote drug adsorption because the contact with intestinal epithelium is kept for extended periods (Fonte et al., 2011; Sogias et al., 2008). It is also reported that chitosan increases the stability of SLNs and minimizes the loss of encapsulated compound, it is an effective permeability enhancer because it reversibly alters the tight junctions and it can provide stealth properties to SLNs together with brij 78 (Bugnicourt & Ladaviere, 2017).

Recently, the combination of chitosan and lipid-based colloids has been considered to produce new entities which gather the advantages of both vectors and to overcome several drawbacks of lipid carriers, such as the sudden release of the drug once they are administered in the human body, the difficulty to incorporate some drugs (Bugnicourt & Ladaviere, 2017).

The purpose of this work was to develop lipid nanoparticulate carriers for the oral administration of Sb by associating the biocompatibility advantages of a lipid matrix, the stealth properties of brij 78 and the mucoadhesive and absorption enhancing effects of chitosan.

Optimized formulations were physically and chemically investigated by Light Scattering techniques (DLS and ELS), electron microscopy (TEM) and chromatographic analyses (HPLC-DAD/FLD). Storage stability and in simulated gastrointestinal conditions were also assessed.

Sb *in vitro* release was studied in different pH media. The ability of SLNs to increase the permeability of Sb was evaluated *in vitro* by using artificial membranes (PAMPA) and Caco-2 cells, after preliminary cytotoxicity studies. The data obtained from a passive diffusion permeability assay and a cell-based test were compared to elucidate the permeation/absorption mechanism of Sb, which further could be correlated to *in vivo* behaviour.

Caco-2 cellular uptake, with or without endocytic inhibitors, was also evaluated, to identify the possible mechanisms of the internalization pathway of the developed SLNs and the effect of the surface coating. Finally, mucoadhesion properties were evaluated by turbidimetry to investigate the effect of chitosan coating.

MATERIALS

Stearic acid, Brij 78 (Polyethylene glycol octadecyl ether), Low Molecular Weight Chitosan, Fluorescein isothiocyanate (FITC, purity $\geq 90\%$, HPLC), Silibin (Sb, purity $\geq 98\%$, HPLC), Sodium chloride (NaCl), Lipase from porcine pancreas, Pepsin from porcine gastric mucosa, Bile salts, Sodium Hydroxide (NaOH), Calcium chloride (CaCl_2), Cholesterol, Lecithin, Phosphate buffered saline (PBS) bioperformance certified, Mucin from porcine stomach, Sodium azide, Chlorpromazine, Indomethacin, Acetone HPLC grade, Acetonitrile (CH_3CN) HPLC grade, Methanol (CH_3OH) HPLC grade, Dimethyl sulfoxide (DMSO) HPLC grade, Acetic acid (CH_3COOH) analytical reagent, Formic acid (HCOOH) analytical grade, Hydrochloric acid (HCl) analytical grade, 1,7-octadiene 98% were purchased from Sigma-Aldrich (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, USA). 96-well Multi-Screen PAMPA filter plates (pore size $0.45\ \mu\text{m}$) were purchased from Millipore Corporation (Tullagreen, Carrigtwohill, County Cork, Ireland). Dialysis kit was from Spectrum Laboratories, Inc. (Breda, The Netherlands).

METHODS

SLNs preparation

SLNs were prepared by means of emulsion/evaporation/solidifying method (Shahgaldian et al., 2003; J. Q. Zhang et al., 2007). Stearic acid, chosen as solid lipid, was completely dissolved in the organic solvent, acetone, by magnetic stirring. The aqueous phase containing brij 78, was heated to $75^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Then, the organic phase was added to the aqueous phase dropwise with fast magnetic stirring to obtain lipid emulsion and then concentrated to induce the acetone evaporation. The translucent emulsion system was added quickly into distilled water at 4°C in an ice bath under fast magnetic stirring to induce SLNs formation. CS-SLNs were obtained using the method described above with slight modifications. After the evaporation of the organic solvent, the hot emulsion was transferred into a 0.2% w/v chitosan solution in acetic acid 1% v/v at 4°C , instead of cold water (Sandri et al., 2017).

Sb-loaded (Sb-SLNs, Sb-CS-SLNs) and FITC-loaded nanoparticles (FITC-SLNs, FITC-CS-SLNs) were prepared by analogous methods adding Sb or the probe to the lipid phase. The final concentration of the components resulted: stearic acid 0.7% w/v, brij 78 1.4% w/v, Sb 0.07% w/v, FITC ($\lambda_{\text{ex}}=492\text{ nm}$, $\lambda_{\text{em}}=518\text{ nm}$, green) 0.035% w/v.

The reaction yields were estimated as the weight percentage of freeze-dried SLNs and CS-SLNs, with respect to the total weight of the substances used for the preparation.

The evaluation of the hydrodynamic diameter, particle size distribution and ζ -potential was performed with using a Zsizer Nanoseries ZS90 (Malvern Instruments, UK). For all measurements, samples were diluted to a suitable concentration with distilled waters. These analyses were performed in triplicate.

Transmission electron microscopy measurements

SLNs and CS-SLNs morphological properties were investigated using transmission electron microscopy (TEM). After dilution with distilled water, samples were deposited on a 200 mesh carbon film-covered copper grid and negatively stained with phosphotungstic acid aqueous solution 1% w/v. After drying nanoparticles were observed with a TEM Jeol 1010 (Tokyo, Japan).

Determination of encapsulation efficiency and recovery

The amount of FITC and Sb encapsulated into SLNs and CS-SLNs was determined as follows: free FITC or Sb was removed through dialysis bag method (cut-off 3.5 kD) for 60 min in 1 L of distilled water, at room temperature. Then SLNs and CS-SLNs were broken by adding 50-fold methanol in an ultrasonic bath for 30 min. The solution was centrifuged for 10 minutes at 11330 x g and the supernatant containing FITC or Sb was analyzed by HPLC. The encapsulation efficiency (EE%) was then calculated using the following equation:

$$EE\% = \frac{C_f}{C_i} \times 100$$

where C_i was the amount of FITC or Sb added in the formulation and C_f was the amount of FITC or Sb encapsulated into SLNs and CS-SLNs.

The recovery percentage (R%) was estimated using the same procedure but without dialysis purification and was calculated using the following equation:

$$R\% = \frac{C_r}{C_i} \times 100$$

where C_r was the amount of FITC or Sb quantified after the destruction of SLNs and CS-SLNs.

HPLC analysis of Sb and FITC

Qualitative and quantitative analyses of Sb were performed using an HP 1100 Liquid Chromatograph with a diode-array-detector. A 150 mm x 4.6 mm i.d., 5 μ m Kinetex EVO, RP18 column was used (All from Agilent Technologies, Santa Clara, CA, USA). The eluents were (A) HCOOH/water pH 3.2, (B) CH₃CN and (C) CH₃OH; the multi-step linear solvent gradient applied was: 0-2 min 5% B and 5% C; 2-6 min 16% B and 16% C; 6-8 min 16% B and 16% C; 8-15 min 26% B and 26% C; 15-17 min 26% B and 26% C; 17-20 min 45% B and 45% C; 20-23 min 5% B and 5% C; equilibration time 7 min; oven temperature 30°C; flow rate 1.0 mL/min. The chromatograms were acquired at 288 nm.

FITC analyses were performed on the same apparatus, with the column used for Sb; eluents were (A) HCOOH/water pH 3.2 and (B) CH₃CN. The gradient applied was: 0-5 min 10-40% B; 5-10 min 40-50% B; 10-12 min 50-55% B; 12-15 min 55% B; 15-18 min 55-90% B; 18-20 min 10% B; equilibration time 5 min; temperature 30°C; flow rate 0.8 mL/min. In this case the chromatograms were acquired at 224 nm.

The linearity range of responses of Sb and FITC dissolved in CH₃OH was determined on five concentration levels with three injections for each level. Both curves showed a coefficient of linear concentration above than 0.999. The detection limit (LOD) and the quantification limit (LOQ) of Sb were estimated investigating the signal-to-noise ratio. LOQ and LOD resulted 10 ng and 7.5 ng, respectively.

Stability studies

The stability of SLNs and CS-SLNs suspension was investigated by monitoring hydrodynamic diameter, particle size distribution and surface properties over 30 days at 4°C. EE% and Recovery% were also checked as previously mentioned over 30 days. Physical stability of SLNs and CS-SLNs was carried out in gastric conditions as follows: 5 mL of nanoparticle suspension were incubated with an equal volume of simulated gastric fluid (0.32% w/v pepsin, 2 g of sodium chloride and 7 mL HCl dissolved in 1 L water and pH adjusted to 2.0 using 1M HCl) in a horizontal shaker for 2h at 37°C. After that, simulated intestinal fluid (lipase 0.4 mg/mL, bile salts 0.7 mg/mL, pancreatin 0.5 mg/mL and calcium chloride solution 750 mM at pH 7.0) was added and the mixture was kept under shaking for further 2 h at 37°C. After the incubation, diameter and particle size distribution were evaluated (Aditya et al., 2013).

In vitro Sb release studies

In vitro release of Sb was investigated using the dialysis bag method in three different conditions: enzyme-free simulated gastric fluid and enzyme-free simulated intestinal medium. For these studies, regenerated cellulose membranes with a molecular cut-off of 3.5 kD were chosen because they retain nanoparticles and allow the diffusion of Sb into dissolution medium. Sb-SLNs, Sb-CS-SLNs or Sb solution (2 mL) were added into dialysis membranes and placed in 200 mL of release medium (SGM and SIM conditions). Thus, the systems were incubated at 37°C with stirring at 150 rpm. At predetermined time intervals (0, 0.5, 1, 2, 4, 6, 9, 24 h) 1 mL of the medium was withdrawn and replaced with the same volume of fresh release medium maintained at 37°C to preserve sink conditions. Sb released at each time interval was quantified using HPLC. *In vitro* release of FITC was also investigated but using the same fluid as the cellular uptake studies as release medium. All experiments were performed in triplicate.

Permeation studies through artificial membranes

Effective permeability values (P_e , cm/s) were determined using the protocol previously reported (Bergonzi et al., 2014). A mixture of lecithin (1% w/v) and cholesterol (0.8% w/v) in 1,7-octadiene was prepared and sonicated to ensure complete dissolution. 10 μ L of the mixture was carefully added to each donor plate well. After that, 0.25 mL of Sb-SLNs, Sb-CS-SLNs and free-Sb were added into the donor plates and 0.25 mL of 5% v/v DMSO in PBS buffer were pipetted into the acceptor compartments. The plate assembly was placed into a sealed container and incubated at room temperature for 4 h. After the incubation, samples were 10-fold diluted with methanol, placed into an ultrasonic bath for 30 min and centrifuged for 10 minutes at 11330 $\times$ g. Sb concentration was evaluated by HPLC. Compounds permeability and recovery were calculated according to the following equations:

$$P_e = C \times -\ln(1 - [Sb]_A / [Sb]_{eq});$$

$$\text{where } C = V_D \times V_A / (V_D + V_A) \times A \times t;$$

$$[Sb]_{eq} = [Sb]_D \times V_D + [Sb]_A \times V_A / (V_D + V_A)$$

$$\text{Recovery (\%)} = [Sb]_D V_D + [Sb]_A V_A / ([Sb]_{D0} V_D) \times 100$$

A = effective filter area (cm^2), V_D and V_A = volumes in the acceptor and the donor compartments (mL), t = incubation time (s), $[Sb]_A$ and $[Sb]_D$ = concentrations of Sb in the acceptor and the donor compartments at time t , $[Sb]_{D0}$ = Sb concentration in the donor compartment at time 0.

In vitro transport studies across Caco-2 cell monolayers

Human colorectal adenocarcinoma (Caco-2) cells were purchased from American Type Cell Culture (ATCC, Rockville, VA) and grown at 37°C in Dulbecco's Modified Essential Medium (DMEM) supplemented with 20% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin, in a humidified atmosphere containing 5% CO₂. The SLNs cytotoxicity was first evaluated by using the Cell Titer 96™ Aqueous One solution cell proliferation (MTS assay). Caco-2 cells were grown on Transwell Greiner culture inserts with a polyethylene terephthalate membrane (1.13 cm² growth surface area and pore size 0.4 µm, Greiner Bio-One, Italia). On the 21st day, the integrity of the monolayers grown on the permeable membrane was assessed using the LY permeability assay (Iacomino et al., 2013). After washing, the monolayers were preincubated for 20 minutes at 37°C with 0.5 and 1.5 mL of the incubation medium, HBSS/HEPES 25 mM in the apical and basolateral sides, respectively. After the preincubation, the medium was removed immediately, and the incubation medium containing FITC, FITC-SLNs or FITC-CS-SLNs, was added to the apical side (0.5 mL). Next, 300 µL samples were taken from the receiver compartment at intervals of 5, 30, and 60 minutes, and replaced with a fresh buffer. At the end of the experiments, the layer integrity was re-evaluated. After extraction with methanol, the concentration of FITC in the transepithelial transport samples was determined using the HPLC method as described above.

Microscopic imaging of cellular uptake

Caco-2 cells were seeded at 1×10^5 /well on histological slides and grown in the culture medium for 24 h at 37 °C. Then, cells were exposed to FITC-SLNs, FITC-CS-SLNs or with a saturated solution of FITC, at 37°C or 4°C, for 1 hour. At the end of the experiment, cells were washed three times with cold PBS, fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 10 min and observed by fluorescence microscopy (Labophot-2 Nikon, Japan). Ten photomicrographs were randomly taken for each sample and fluorescence was measured using ImageJ 1.33 image analysis software (<http://rsb.info.nih.gov/ij>).

Transport pathways

To identify the possible transport mechanism of SLNs and CS-SLNs by the intestinal cells, Caco-2 cells (1×10^5) were exposed for 1 h to FITC-SLNs and to FITC-CS-SLNs diluted 1:160 and 1:120, respectively, into the culture media or with a saturated solution of FITC, in the presence/absence of endocytic inhibitors. Control cells were exposed to FITC-SLNs and FITC-CS-SLNs without any pre-treatment with any agent and their uptake was assumed as 100%. A second group of cells was pre-treated with 1 μ M sodium azide at 37 °C for 30 min and then with the SLNs, to evaluate the uptake under ATP-depletion. A third group of cells was pre-treated with 15 μ M chlorpromazine (a clathrin blocker) for 30 min followed by incubation with SLNs. A fourth group of cells was pretreated with 25 μ M of indomethacin (a caveolin-dependent endocytosis inhibitor) for 30 min; finally, a fifth group of cells was maintained at 4°C during the SLNs exposure, to observe the effect of low temperature, a general metabolic inhibitor. At the end of the treatments, the amount of FITC was measured on cellular lysate. FITC was extracted from the lysate with methanol and quantified by HPLC as described above.

Mucoadhesion properties

The mucoadhesion properties of SLNs and CS-SLNs in the presence of mucin were assessed by means of the turbidimetric method (Bonferoni et al., 2010). An equal volume of nanoparticles suspension and mucin aqueous solution at different concentrations (0.25, 0.75, 1.0, 1.5 and 2.0% w/v) were mixed. Then, the resulted samples were vortexed for 1 min and incubated in a water bath for 1h at 37°C. The turbidity of the mixtures was spectrophotometrically evaluated at $\lambda = 500$ nm. The absorbencies of individual mucin and nanoparticles suspensions were also recorded. The interaction between mucin and SLNs was calculated using the following equation:

$$\Delta A = A - A_{\text{theor}}$$

where ΔA is the absorbance difference, A is the effective absorbance of SLNs-mucin and CS-SLNs-mucin mixtures and A_{theor} represents the sum between the individual absorbance of mucin and SLNs suspensions. If $\Delta A = 0$, no interactions occur, while if $\Delta A \gg 0$ there was a strong interaction between nanoparticles and mucin.

Statistical analysis

Experiments were repeated three times and results expressed as a mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

Nanoparticles characterization

Sb, as others plant-derived molecules, is characterized by poor water solubility and low bioavailability after oral administration. In this work, SLNs and CS-SLNs were developed and characterized to increase its solubility and favour its intestinal absorption (Table 1).

Sample	Size (nm)	PdI	ζ -potential (mV)	EE (%)	Recovery (%)	Yield (%)
SLNs	148 $\pm$ 9	0.25 $\pm$ 0.04	-43.2 $\pm$ 0.1	-	-	96.2 $\pm$ 0.2
FITC-SLNs	190 $\pm$ 9	0.32 $\pm$ 0.08	-46.4 $\pm$ 0.1	90 $\pm$ 1	98.1 $\pm$ 0.1	-
Sb-SLNs	169 $\pm$ 12	0.27 $\pm$ 0.04	-48.1 $\pm$ 7.7	96 $\pm$ 2	99.2 $\pm$ 0.1	99.1 $\pm$ 0.1
CS-SLNs	224 $\pm$ 2	0.31 $\pm$ 0.01	+33.4 $\pm$ 4.2	-	-	98.2 $\pm$ 0.1
FITC-CS-SLNs	240 $\pm$ 14	0.34 $\pm$ 0.10	+29.3 $\pm$ 3.6	91 $\pm$ 5	99.0 $\pm$ 0.1	-
Sb-CS-SLNs	262 $\pm$ 16	0.33 $\pm$ 0.05	+40.1 $\pm$ 1.2	97 $\pm$ 1	99.3 $\pm$ 0.1	99.3 $\pm$ 0.1

Table 1. Physical and chemical characterization of empty SLNs and CS-SLNs and Sb or FITC loaded nanoparticles.

Results are expressed as means $\pm$ standard deviation of at least three experiments.

Empty SLNs and Sb-SLNs, made of stearic acid as solid lipid and brij 78 as surfactant exhibited an average diameter below 170 nm and good properties in terms of homogeneity with PDI below 0.3. High ζ -potential values indicated the stability of the formulations. Indeed, high electrostatic repulsive forces prevent nanoparticles aggregation. Brij 78 was chosen as stealth agent; it is derived from stearic acid covalently grafted with PEG 1000. These hydrophilic chains lead to an improvement of blood residence time, suppressing the binding of serum proteins and opsonic factors.

The ζ -potential values obtained for SLNs and Sb-SLNs were -43 and -48 mV, respectively. Thus, the encapsulation of Sb did not also affect the superficial charge of the nanoparticles.

CS-coated formulations (CS-SLNs and Sb-CS-SLNs) exhibited larger particle size than uncoated SLNs and positive surface charge, providing evidence of CS coating. Such increase of the mean particle size is attributable to the chitosan coating around the surface of SLNs (Fonte et al., 2011). The interaction between CS and SLNs is mainly electrostatic but also hydrogen bonds between polar groups of the lipid and CS chains occurred. ζ -potential influences the interactions with gastrointestinal mucus, in particular positive surface charge improves this interaction. Moreover, all SLNs formulations show a high absolute ζ -potential charge and therefore they are considered physically stable due to the electrostatic repulsions between nanoparticles.

Both in the case of Sb-SLNs and Sb-CS-SLNs formulations, Sb determined a slight increase of particle size, but didn't affect the homogeneity and the surface charge (Fonte et al., 2011; Vieira et al., 2018). Nevertheless, chitosan-coated SLN are within the appropriate size range for intestinal absorption, lower than 500 nm, as desirable to increase the contact with the intestinal mucosa and to facilitate the permeation (Florence, 2004; Fonte et al., 2011).

The processes yield, calculated on the freeze-dried formulations, were around 100%. The EE% and recovery% resulted very high; this means that there is a strong affinity of Sb to lipid matrix and the surface modifications did not impair the EE%. Furthermore, high EE% might reduce gastrointestinal degradation of Sb, which could be absorbed and reach the target site after oral administration. Furthermore, high EE% can avoid of the first pass metabolism, a major limitation of oral drug delivery, because high amount of absorbed drug may pass through the lymphatic transport (Venishetty et al., 2012).

TEM micrographs provided information on morphology and dimensions of nanoparticles: all the formulations had a spherical structure with a diameter like that obtained through DLS analyses. Moreover, TEM shows the presence of CS adsorbed on the surface of SLNs, as grey shell around nanoparticles (Fonte et al., 2011; Bugnicourt & Ladaviere, 2017) (Figure 1). It is also possible to notice that CS-SLN are isolated, maintaining most of the properties acquired before coating.

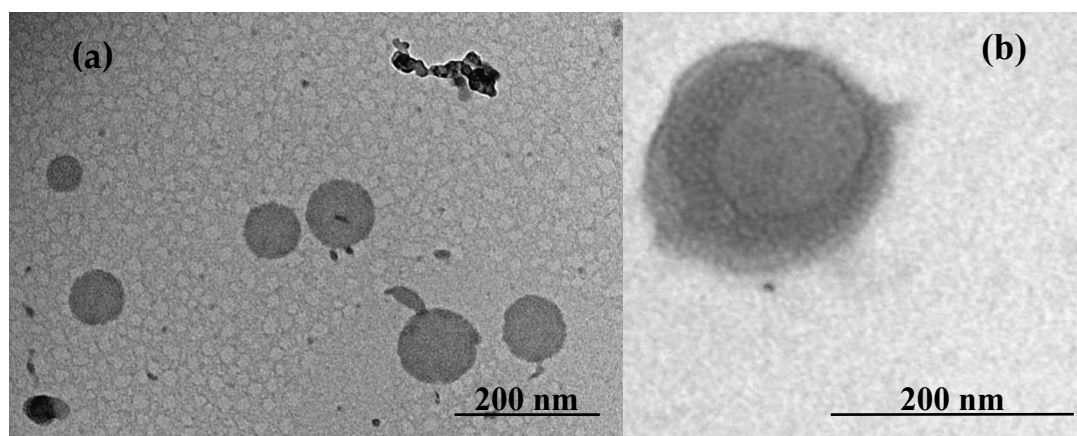


Figure 1. TEM images of SLNs (a) and CS-SLNs (b).

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Chemical and physical storage stability

After 4 weeks at 4°C, both Sb-SLNs and Sb-CS-SLNs suspensions were chemically and physically stable. No changes in size and homogeneity occurred (Table 2). The ζ -potential was monitored up to 30 days of storage, and no significant evolution was found. Furthermore, EE% and recovery%, calculated by HPLC, were not decreased during the storage period. Thus, developed formulations could be able to prevent the degradation of the incorporated compound. This represents an important result, considering that in many cases chemical and physical instabilities were observed when SLNs were stored as aqueous suspension due to lipid crystallization, polymorphic transformations, aggregation phenomena and hydrolysis processes.

Sample	Size (nm)		PdI		ζ -Potential (mV)		EE%		Recovery%	
	0 day	30 days	0 day	30 days	0 day	30 days	0 day	30 days	0 day	30 days
Sb-SLNs	172 $\pm$ 3	183 $\pm$ 6	0.28 $\pm$ 0.05	0.31 $\pm$ 0.03	-47.2 $\pm$ 3.4	-47.5 $\pm$ 5.6	96.6 $\pm$ 1.6	96.0 $\pm$ 3.4	99.2 $\pm$ 0.1	99.5 $\pm$ 0.1
Sb-CS-SLNs	263 $\pm$ 5	266 $\pm$ 1	0.30 $\pm$ 0.02	0.30 $\pm$ 0.01	+43.1 $\pm$ 6.5	+40.5 $\pm$ 2.3	97.0 $\pm$ 0.5	96.0 $\pm$ 2.1	99.3 $\pm$ 0.1	99.0 $\pm$ 0.1

Table 2. Physical and chemical stability of Sb-SLNs and Sb-CS-SLNs during 30 days at 4°C.

Results are expressed as means $\pm$ standard deviation of at least three experiments.

Stability in simulated gastrointestinal fluids

One of the aims of this work was the development of drug delivery systems able to improve the transport of Sb through the gastrointestinal tract. As previously reported, SLNs are absorbed mostly intact through the intestinal epithelium. For this purpose, the physical stability of the formulations in SGIM was determined by subjecting them to simulated gastric digestion at pH 2.0 in the presence of pepsin, followed by simulated intestinal digestion in the presence of the pancreatin-lipase-bile salts mixture. Indeed, the preservation of particle size after oral administration is a crucial factor to reach the systemic circulation by absorptive enterocytes.

After 2 h of incubation in gastric conditions followed by 2 h of incubation in simulated intestinal environment, SLNs and CS-SLNs were stable and no aggregation or degradation phenomena occurred (Table 3). This stability in acidic pH and in intestinal conditions may be due to steric stabilization effect by non-ionic surfactant, brij 78, which is indifferent to flocculation and coalescence due to its molecular structure; in addition, acid stable lipid such as stearic acid and chitosan coating might have also contributed to the stability of nanoparticles in SGIM.

Sample	Initial	Stomach (pH 2)	Intestine (pH 7)
SLNs	148 ± 9	152 ± 2	161 ± 4
CS-SLNs	224 ± 2	225 ± 3	226 ± 2

Table 3. Physical stability of SLNs and CS-SLNs in simulated gastrointestinal fluids.
Results are expressed as means ± standard deviation of three experiments

In vitro release

After demonstrating physical stability of SLNs and CS-SLNs in SGIM, *in vitro* release studies were performed in different pH media without enzymes: SGM (pH 2) and SIM (pH 7) conditions. The *in vitro* Sb release profiles of SLNs was shown in Figure 2 and 3. In SGM, Sb release from both SLNs formulations was negligible (Figure 2). This result suggests that SLNs prevent gastric degradation of entrapped molecule and may be promising carriers to delivery large amount of Sb to the intestine. On the other hand, both SLNs showed similar release pattern in SIM (Figure 3). The percentage of Sb released remained low after 24 h and at the end of the experiments the release percentage of Sb was 11.45% for Sb-SLNs and 4.20% for Sb-CS-SLNs. The presence of CS coating reduces the release rate and it suggests that the drug leakage during *in vitro* and *in vivo* delivery would be minimal (Xu et al., 2013).

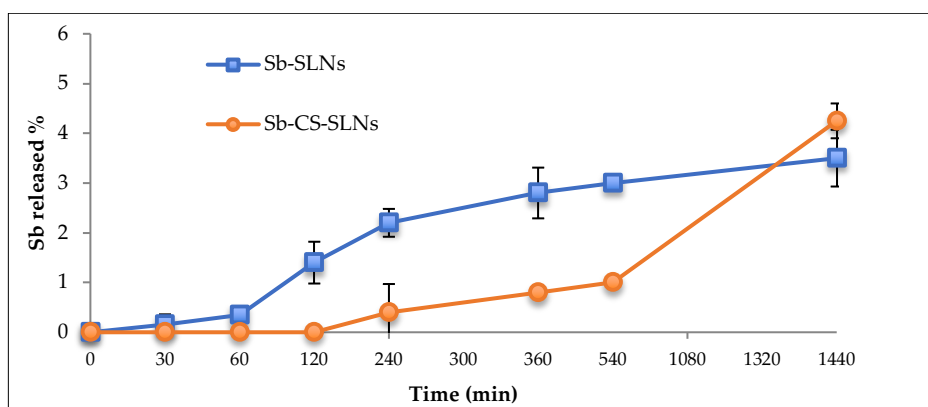


Figure 2. *In vitro* release of Sb from Sb-SLNs and Sb-CS-SLNs in SGM. Results are expressed as means $\pm$ standard deviation of three experiments.

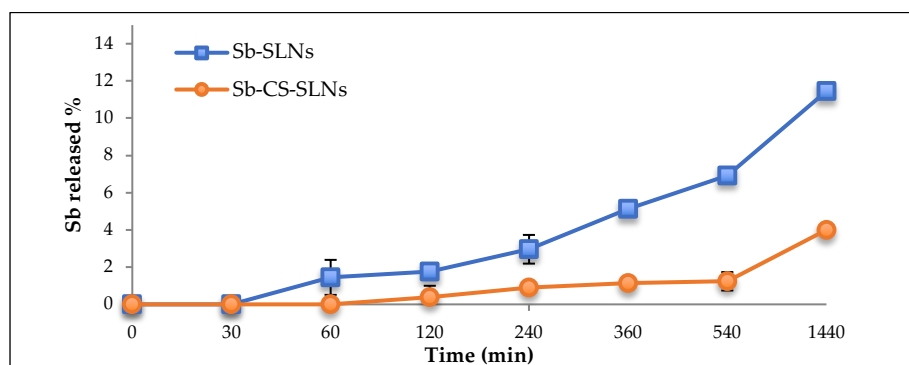


Figure 3. *In vitro* release of Sb from Sb-SLNs and Sb-CS-SLNs in SIM. Results are expressed as means $\pm$ standard deviation of three experiments.

SLNs demonstrated promising potential to prevent burst release and degradation of Sb which remained entrapped into nanoparticles and could be absorbed and reach the target site after oral administration. In fact, the percentage of Sb released from a Sb solution gets to the 100% in 1 h both in SGM and SIM conditions.

As previously reported, SLNs show the ability to achieve a controlled release of encapsulated hydrophobic compounds, due to their solid status at room or body temperature and strong hydrophobic interactions in this case with lipophilic silybin. Brij and chitosan coating was beneficial to further slower the release rate (Dharmala et al., 2008; Luo et al, 2015).

PAMPA studies

The PAMPA is one of the most used *in vitro* methods to estimate transcellular passive permeability.

It is a non-cell-based permeability model but is considered robust, reproducible and fast (Kansy et al., 1998). The assay resulted useful not only in the preformulation studies of single molecules but in also for complex matrices such as plant extracts (Petit et al., 2016) and compounds loaded into nanoformulations. Considering that most compounds reach the blood stream by passive mechanism, PAMPA has been proposed as helpful complement to Caco-2 assay to predict the oral absorption of Sb. The effective permeability (P_e) values are reported in Table 4.

Sample	Sb $P_e \times (10^{-6})$ (cm/s)
Free Sb	3.6 ± 0.1
Sb-SLNs	25 ± 8
Sb-CS-SLNs	27 ± 8

Table 4. Effective permeability coefficients (P_e) of Sb in PAMPA.

Recovery was > 80% (A recovery > 80% is required for an acceptable *in vitro* prediction).

Results are expressed as means $\pm$ standard deviation of at least three experiments.

The maximum P_e values were found after 4 h of incubation. Passive permeation of Sb was improved considerably (7/7.5-fold higher) when are formulated, respect to aqueous solution. As required for an acceptable permeation prediction (Palmgren et al., 2006; Heikkinen et al., 2009; Broeders et al., 2012) a recovery above 80% was achieved for free Sb and Sb loaded into SLNs. Moreover, this means there was neither relevant Sb membrane retention nor binding to the surface of the filter. Thus, the obtained results confirm that developed formulations represent a successful attempt to ameliorate passive permeation of Sb.

In vitro cell culture experiments

The cytotoxicity of SLNs was first investigated to select the concentration for transport studies identifying as appropriate dilution, 1:160 for the Sb-CS-SLNs and 1:120 for the Sb-SLNs. This is a good result considering that oral formulations undergo a dilution from 1:200 to 1:1000 with biological fluids after oral administration. The biocompatibility of chitosan is well known and, its presence in the formulation, reduced the cytotoxicity of the SLN.

Before the transport experiments, Lucifer Yellow (LY) was used to prove the absence of monolayer cleavage. The LY passage resulted less than 2%, confirming the absence of leaky layer. Transport experiments were carried out with formulations containing FITC, a probe not able to cross the Caco-2 monolayer, as evidenced by P_{app} values (Table 5). In fact, free-FITC was not detected into basolateral chamber. On the contrary, both SLNs and CS-SLNs enhanced FITC permeation. As in the case of PAMPA, recovery was estimated, resulting above 80%, as required for acceptable *in vitro* permeation prediction.

Furthermore, a probe release study was also performed, to evaluate the integrity of the formulations permeated across the monolayer. The results revealed that only 3% of the probe was released, indicating that FITC remains entrapped into SLNs. These results support the hypothesis that carriers can cross intestinal

epithelium and represent promising delivery systems to increase the absorption and the transport of lipophilic molecules into the systemic circulation. Although these experiments did not consider the important pharmacokinetic parameters, obtained results confirmed that different models, such as PAMPA and Caco-2 cells, could be synergistically used for the correct evaluation of drugs permeability and absorption.

Sample	FITC $P_{app} \times (10^{-6})$ (cm/s)
Free-FITC	ND
FITC-SLN _s	20 ± 6
FITC-CS-SLN _s	15 ± 5

Table 5. Apparent permeability coefficients (P_{app}) of Sb in Caco-2 assay. Results are expressed as means $\pm$ standard deviation of at least three experiments.

Microscopic imaging of cellular uptake revealed that both formulations were internalized into the cells. Indeed, after 1 h of incubation at 37°C, green spots related to both SLNs were observed into cytoplasm of Caco-2 cells (Figure 4, panel A and C) and the use of FITC-CS-SLNs allows the higher uptake (Figure 4, panel C). On the contrary, no fluorescence was detected after incubation with free-FITC indicating that the model dye, by itself, was not internalized (Figure 5). The marked reduction of cellular uptake at 4°C suggested also that the endocytic uptake requires energy, for both formulations (Figure 4, panel B and D).

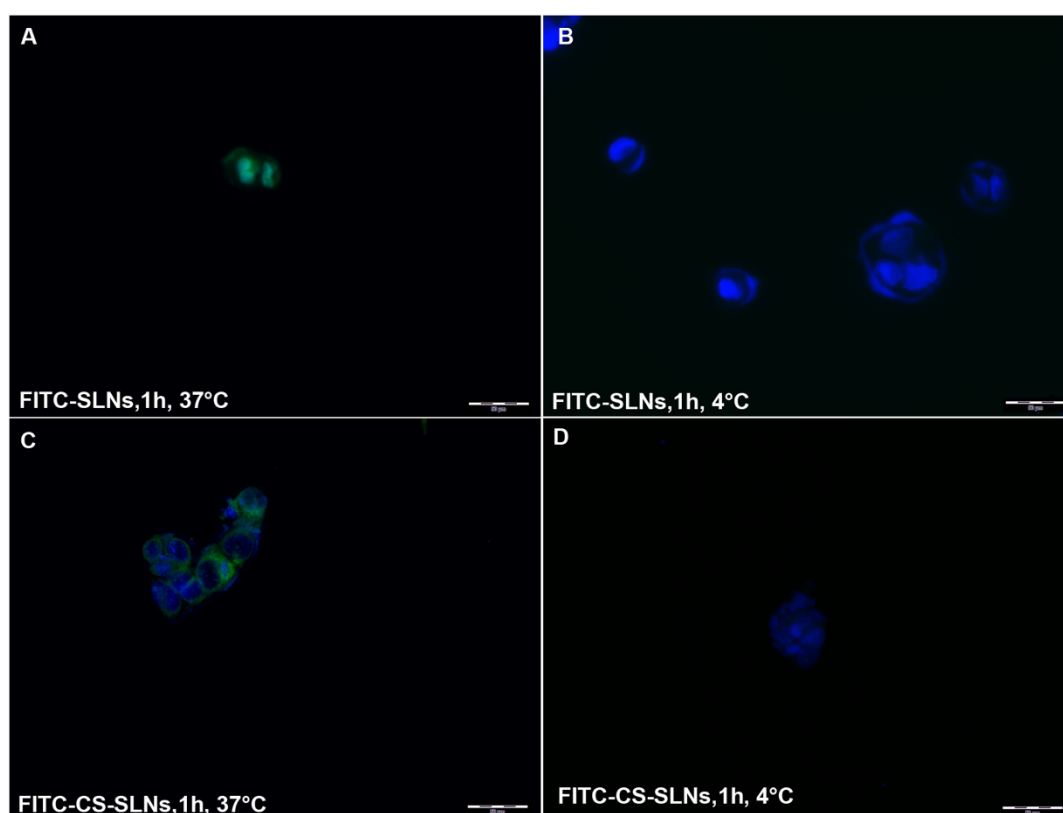


Figure 4. Cellular uptake. Panel A: FITC-SLNs, 1h, 37°C; Panel B: FITC-SLNs, 1h, 4°C; Panel C: FITC-CS-SLNs, 1h, 37°C; Panel D: FITC-CS-SLNs, 1h, 4°C.

Nuclei were stained with DAPI.

Scale bar: 50 μm.

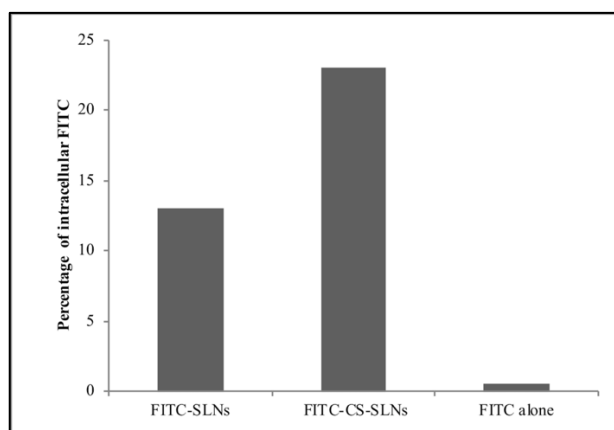


Figure 5. Percentage of intracellular FITC in Caco-2 cells exposed to FITC-SLNs or FITC-CS-SLNs loaded with 5 mg of FITC or FITC alone, after 1 hour at 37°C. Results are expressed as means $\pm$ standard deviation of at least three experiments

In addition to qualitative uptake studies, the percentage of FITC internalized was calculated. Low temperature, i.e. 4°C, and sodium azide are general metabolic inhibitors. Setting up the internalization experiments at 4°C and in presence of sodium azide, cellular uptake of both formulations resulted decreased, which confirmed that active processes were involved in the internalization of nanoparticles (Figure 6).

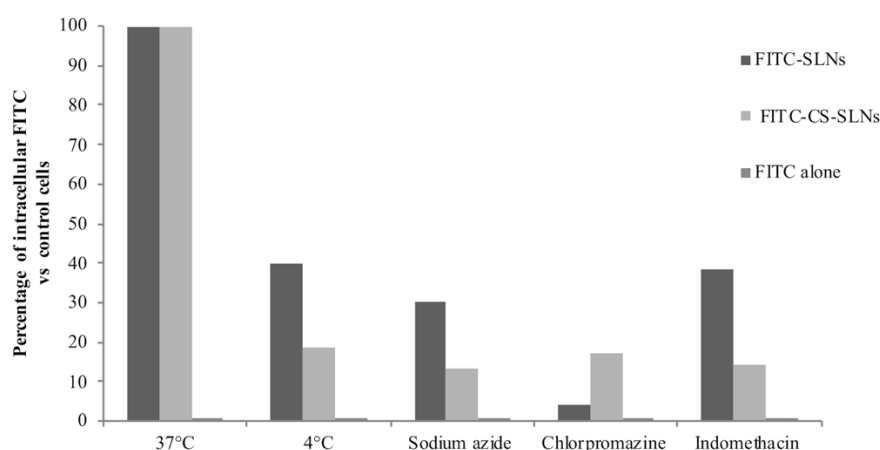


Figure 6. Percentage of intracellular FITC in Caco-2 cells exposed to FITC-SLNs or FITC-CS-SLNs or FITC alone and in the presence of endocytic inhibitors. Results are expressed as means $\pm$ standard deviation of at least three experiments

Moreover, to identify the possible internalization pathways, these experiments were performed also in the presence of chlorpromazine, a clathrin-dependent endocytosis inhibitor, and indomethacin, a caveolin-dependent endocytosis inhibitor. The uptake in the presence of inhibitors decreased for both formulations, suggesting the complexity of the endocytic uptake of SLNs, which requires energy and involves all the mechanisms explored. In particular, the results in the presence of chlorpromazine suggest that for FITC-SLNs the uptake is more clathrin-dependent than for the FITC-CS-SLNs.

Mucoadhesion properties

Mucoadhesion is an important factor for the development of drug delivery systems for oral administration. The strong mucoadhesion suggests intimate contact with absorption site, and it ensures the effective absorption. Several interactions are involved in the mucoadhesion, such as hydrogen, ionic, van der Waals, and hydrophobic interactions. Since mucin is a negatively charged protein, the positively charged molecules, such as chitosan, have a strong affinity. The mucoadhesion was determined by turbidity analysis method (Bonferoni et al., 2010). To differentiate the mucoadhesion of SLNs and CS-SLNs, performances were evaluated after incubation of nanoparticles with different concentrations of mucin, in the range of 0.25%-2% w/v.

Turbidimetric measurements showed that there was a strong interaction between mucin and CS-SLNs at mucin concentrations above than 1% w/v (Table 6). This is probably due to the electrostatic interaction between positive charges of chitosan and negative charges of mucin which results in adsorption of mucin around the nanoparticles.

Best mucoadhesive properties are noticeable with mucin greater than 1% w/v, suggesting that optimal coverage of nanoparticles occurs at this concentration (Sandri et al., 2017). CS-SLNs present higher values of ΔA with respect to the uncoated SLNs, thus displaying higher mucoadhesion performance, due to the presence of chitosan. Seemingly, obtained values indicated that there was also a little interaction between mucin and uncoated SLNs, in fact the slope of the curve was like that of the mucin alone (Figure 7). This indicates that the turbidity increase was due only to the increase of mucin concentration. On the contrary, in the case of CS-SLNs, at the concentration greater than 1%, the slope was higher than that of mucin and SLNs meaning that a strong interaction occurred (Rossi et al., 2000; Bonferoni et al., 2010). These results proved that the presence of chitosan coating on the surface enhances SLNs adhesion properties resulting in an improvement of drug absorption.

Mucin % w/v	ΔA SLNs	ΔA CS-SLNs
0.25	0.41	0.47
0.75	0.80	0.80
1	0.94	1.12
1.5	0.96	3.42
2	1.75	4.87

Table 6. Absorbance difference in presence of different concentrations of mucin.

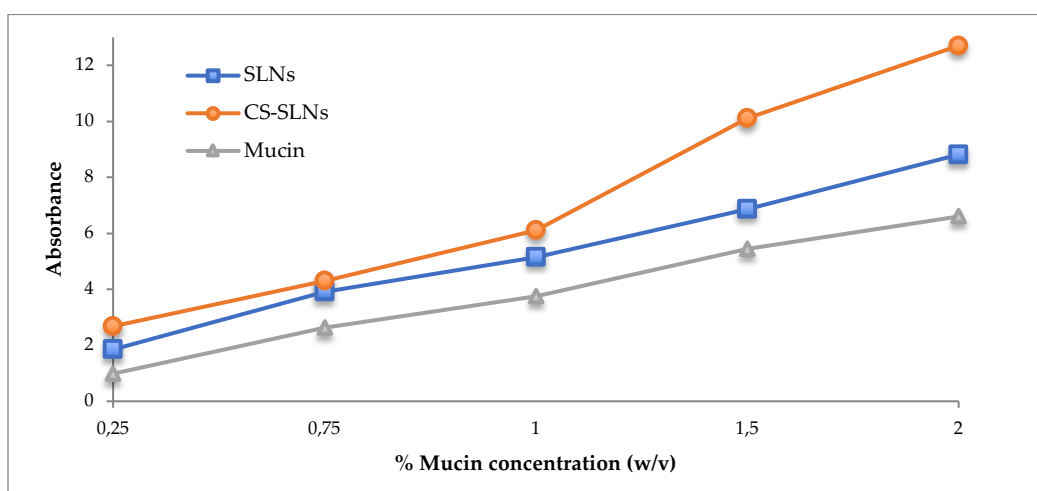


Figure 7. Values of absorbance of SLNs, CS-SLNs and mucin alone.

CONCLUSIONS

SLNs and chitosan-coated SLNs have been proposed in this work as drug delivery systems to enhance the solubility and intestinal permeability of lipophilic Sb. Stearic acid and brij 78 were chosen as solid lipid and non-ionic surfactant, respectively. Based on the obtained results in terms of size, homogeneity, ζ -potential and EE% developed formulations could be orally administered. The systems showed excellent chemical and physical stability during storage at 4°C until four weeks. Furthermore, the formulations were also stable after incubation into simulated gastrointestinal environment. *In vitro* release indicated that SLNs may prevent burst release and gastric degradation of Sb. Moreover, the slow release of Sb suggests homogeneous entrapment of the compound throughout the lipid matrix.

Permeation studies revealed that the formulations were successful in enhancing the permeation of Sb. Cellular uptake into Caco-2 cells of both formulations was demonstrated. The results suggested a predominant active endocytic process in the absorption of SLNs. The cellular uptake was energy-dependent and significantly reduced in the presence of chlorpromazine and indomethacin suggesting that SLNs could be internalized via clathrin- and caveolin-mediated endocytosis. Thus, based on the above results, SLNs could be used as alternative oral formulation to enhance bioavailability and, finally, therapeutic effects of Sb. Moreover, chitosan coating significantly improves mucoadhesion properties of SLNs. Together with the excellent stability, strong mucoadhesive property, and slow release, chitosan coated SLNs demonstrated promising potential to enhance absorption of hydrophobic Sb after oral administration. Future studies will be aimed to evaluate *in vivo* how chitosan coating influences the biodistribution of SLNs.

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RESEARCH ARTICLE

Solid Lipid Nanoparticles and Chitosan-coated Solid Lipid Nanoparticles as Promising Tool for Silybin Delivery: Formulation, Characterization, and *In vitro* Evaluation

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Current Drug Delivery.

NANOSTRUCTURED LIPID CARRIERS OF SILYMARIN: *IN VITRO* AND *IN VIVO* STUDIES

INTRODUCTION

Lipid-based nanocarriers, such as micro- and nanoemulsions, solid lipid nanoparticles, and nanostructured lipid carriers, represent successful examples of nanoformulations applied to overcome the limitations of natural compounds (Bilia et al., 2017; Bilia et al., 2018). They can ameliorate solubility, stability, permeation and bioavailability. Lipid-based drug delivery systems are composed by lipids that are also absorption enhancers (Porter et al., 2008; Chakraborty et al., 2009) and can be developed in small particle sizes ranging from micro- to nanometers (Jia, 2005; Pouton, 2006) to increase the absorption in the hydrophilic environment of the gastrointestinal tract.

Among lipid-based delivery systems, nanostructured lipid carriers (NLCs) offer many advantages, such as long-term stability, increased bioavailability of encapsulated active ingredient, possibility to obtain a controlled or targeted release, versatility in encapsulating both lipophilic and hydrophilic drugs, and high efficiency of encapsulation (Beloqui et al., 2016). NLCs combine two features: they are colloidal carriers of submicron size and they are composed of a solid-lipid core consisting of a mixture of solid lipids blended with a liquid lipid to form a nanosized unstructured matrix and an aqueous phase containing a surfactant or a mixture of surfactants. The partially solid nanostructure enhances the stability of entrapped bioactive, enables high loading capacities, and offers sustained release profiles. The lipid matrix is made of biocompatible and biodegradable materials, which decrease the risk of acute or chronic toxicity. All the components are commercially available and/or approved by regulatory agencies (Muller et al., 2000; Strickley, 2004; Nanjwade et al., 2011).

Silymarin (SLM) is one of the oldest traditional herbal medicines used to treat different disorders. SLM is mainly composed of four flavonolignan isomers:

silybin, isosilybin, silychristin and silydianin (Kvasnicka et al., 2003; Ho et al., 2007). Hepatoprotective effects of SLM have been demonstrated. It is mildly toxic and exerts significant anti-carcinogenic and anti-inflammatory effects. Recently, its beneficial effect in type 2 diabetes patients has been reported and many articles demonstrated its ability to decrease both fasting and mean daily glucose, triglycerides and total cholesterol levels (Huseini et al., 2006; Voroneanu et al., 2016). Antidiabetic effect of SLM is attributed to inhibition of gluconeogenesis in the liver and decrease of glucose-6 phosphatase activity (Guigas et al., 2007-2008). However, it is a low water soluble and low permeable biopharmaceutic class IV type compound that faces stipulated requirements in delivery design. The aim of the study was to investigate the possibility to deliver lipophilic SLM into NLCs for the preparation of oral dosage form.

NLCs were characterized in terms of average diameter, polydispersity, ζ -potential, morphology, and physical and chemical stability. The freeze-drying process was also considered to increase long-term storage stability of the formulation.

Storage stability and in simulated gastrointestinal conditions were also assessed. SLM *in vitro* release was studied in different pH media. The release results were applied to define the kinetic and mechanism of SLM release from the NLCs.

The ability of NLCs to ameliorate the permeability of SLM was evaluated *in vitro* by using parallel artificial membrane permeability assay (PAMPA). Fluorescent nanoparticles were also prepared to verify the capacity of NLCs to enhance the permeability across Caco-2 cells layer. Preliminary cytotoxicity studies were conducted to confirm the optimized biopharmaceutical properties of the lipid carrier. The data obtained from the *in vitro* tests were compared to elucidate the permeation/absorption mechanism of SLM, which further could be correlated to *in vivo* behaviour. Caco-2 cellular uptake, with or without endocytic inhibitors, was also evaluated, to identify the possible mechanisms of the internalization pathway of the developed NLCs.

Furthermore, after preliminary toxicological studies, the formulations were studied *in vivo* in a streptozotocin-induced diabetic mouse model. Disease Metabolic syndrome was also induced feeding the animals with a high-fat diet.

MATERIALS

Stearic acid, Brij S20 (or brij 78, Polyethylene glycol octadecyl ether), Fluorescein isothiocyanate (FITC, purity 90%, HPLC), Silymarin (SLM), Silybine (Sb, purity 98%, HPLC), Sodium chloride (NaCl), Lipase from porcine pancreas, Pepsin from porcine gastric mucosa, Bile salts, Sodium Hydroxide (NaOH), Calcium chloride (CaCl_2), Cholesterol, Lecithin, Phosphate buffered saline (PBS) bioperformance certified, Mucin from porcine stomach, Sodium azide, Chlorpromazine, Indomethacin, DL- α -Tocopherol acetate (Vitamin E), D-Mannitol, D(+)-Glucose anhydrous, Acetone high performance liquid chromatography (HPLC) grade, Acetonitrile HPLC grade, Methanol HPLC grade, Dimethyl sulfoxide (DMSO) HPLC grade, Formic acid analytical grade, Hydrochloric acid (HCl) analytical grade, and 1,7-octadiene 98%, Dichloromethane (CH_2Cl_2) were purchased from Sigma-Aldrich (Milan, Italy). Capryol 90 (Propylene glycol monocaprylate), Lauroglycol 90 (Propylene glycol monolaurate), Labrafac PG (Propylene glycol dicaprylocaprate), Labrafac WL 1349 (Triglycerides medium-chain), Labrafil M 1944 CS (Oleoyl polyoxyl-6 glycerides), and Precirol ATO 5 (Glyceryl palmitostearate) were a gift of Gattefossé (Saint Priest, France). Oleic acid was purchased from Carlo Erba Spa (Milan, Italy). Hemp oil and borage oil were from Galeno srl (Comeana, Prato, Italy). Water was purified by a Milli-Qplus system from Millipore (Milford, CT, USA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, PA, USA). The 96-well Multi-Screen PAMPA filter plates (pore size 0.45 μm) were purchased from Millipore Corporation (Tullagreen, Carrigtwohill, County Cork, Ireland). Dialysis kit was from Spectrum Laboratories, Inc. (Breda, The Netherlands).

METHODS

Screening of Liquid Lipids

The ability of different liquid lipids (oils) to solubilize SLM was evaluated in order to increase the encapsulation efficiency the NLCs (Chakraborty et al., 2009). The solubility of SLM was determined by adding an excess amount of SLM into an exact volume of each selected vehicle while keeping at 500 rpm for 24 h at room temperature. Further, the samples were centrifuged at 14,000 rpm for 10 min. The supernatants were diluted with methanol or with methanol/dichloromethane (3:2 v/v or 1:1 v/v) mixture and finally analyzed with HPLC consisting of an HP 1100 Liquid Chromatograph with a diode-array-detector (DAD) and controlled with a HP 9000 workstation (Agilent Technologies, Santa Clara, CA, USA). An RP-C18 Luna Omega Polar (150 mm × 3 mm, 5 µm) analytical column was used (Agilent Technologies, Santa Clara, CA, USA). The mobile phases were: (A) formic acid/water pH 3.2; (B) acetonitrile; and (C) methanol with 0.4 mL/min flow rate. The multi-step linear solvent gradient applied was: 0–2 min 10% B and 10% C; 2–6 min 15% B and 22% C; 6–11 min 20% B and 30% C; 11–16 min 30% B and 40% C; 16–18 min 30% B and 40% C; 18–20 min 40% B and 40% C; 20–23 min 40% B and 40% C; 23–27 min 10% B and 10% C; equilibration time 8 min; oven temperature 25 °C. The chromatograms were registered at a wavelength of 288 nm. The calibration curve was prepared using standard silibinin, dissolved in methanol from a concentration range of 0.001–0.100 µg/µL.

Preparation of NLCs

Two different NLCs formulations were prepared by the emulsion-evaporation method at high temperature and solidification at low temperature (J. Q. Zhang et al., 2007). The lipid phase, made of stearic Acid (80 mg, solid lipid, SA) and Capryol 90 (20 mg, liquid lipid) or cetyl palmitate (80 mg, solid lipid, CP) and Lauroglycol 90 (20 mg, liquid lipid), was dissolved into 5 mL of acetone and heated under magnetic stirring. The resulted organic solution was added dropwise into the aqueous phase containing brij 78 (200 mg in 30 mL of distilled water for NLCs-SA and 300 mg in 30 mL for NLCs-CP) at 1000 rpm and 75 °C to induce the acetone evaporation. Further, the concentrated emulsion was quickly added into an equal volume of cold distilled water in an ice bath under fast stirring at 1000 rpm for 1 h to induce NLCs formation. The obtained NLCs were harvested and stored at 4 °C. The final concentrations of stearic acid, Capryol 90 and brij 78 were 0.57% w/v, 0.14% w/v and 1.43% w/v, respectively, while, in the case of NLCs-CP the final concentrations of the components were: CP 0.57% w/v, Lauroglycol 90 0.14% w/v and brij 78 2.14% w/v.

Preparation of SLM-Loaded NLCs and FITC-Loaded NLCs

The SLM-loaded NLCs (SLM-NLCs) and the fluorescent NLCs (FITC-NLCs) were prepared according to the method previously described (J. Q. Zhang et al., 2007). Fluorescein isothiocyanate (FITC) ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 518 \text{ nm}$, green) or SLM were added to lipid phase to obtain a final concentration of 0.04% w/v for FITC and 0.29% w/v for SLM. Further, the formulations were hermetically sealed and stored at 4°C, protected from light.

In addition, the SLM-NLCs samples were divided into three parts: one of these was frozen with liquid nitrogen and then moved to the freeze-drier (Schwarz & Mehnert, 1997). The other two were freeze-dried after the addition of D-Glucose anhydrous (10% w/v) or D-Mannitol (10% w/v) as cryoprotective agents. The drying time was 24 h. The reaction yields were calculated as the weight percentage of freeze-dried formulations, with respect to the total weight of the substances used for the preparation.

Particle Size, Polydispersity Index and ζ -Potential

The evaluation of the particle size and the polydispersity index (Pdl) was performed by dynamic light scattering using a Zsizer Nanoseries ZS90 (Malvern Instrument, Worcestershire, UK) at a fixed angle of 90° and at 25 °C. The ζ -potential, indicating the electric charge on the NLCs surface and the physical stability of the formulations, was calculated using the Helmholtz–Smoluchowski's equation using the same instrument. To avoid multiple scatterings of the light, for all measurements, samples were diluted to a suitable concentration with distilled water. The analyses were performed in triplicate.

Transmission Electron Microscopy

The shape and the surface structure of NLCs were investigated by transmission electron microscope (TEM, Jeol 1010, Tokyo, Japan). The samples were opportunely diluted with distilled water, then were dropped on a 200 mesh carbon film-covered copper grid and negatively stained with phosphotungstic acid aqueous solution (1% w/v). After drying, the grid containing the samples were observed with the TEM.

Determination of Encapsulation Efficiency and Recovery

Dialysis bag method was applied to remove free SLM (or FITC) from the formulations. The bag was inserted for 1 h in 1 L of distilled water, with the aim to determine the encapsulation efficiency of the NLCs. Then, the samples were diluted with methanol and sonicated in an ultrasonic bath for 30 min to extract SLM (or FITC). The resulted mixtures were centrifuged for 10 min at 14,000 rpm and analyzed by HPLC (Righeschi et al., 2014). FITC analyses were performed on the same apparatus, with the column used for SLM. The eluents were: (A) formic acid/water pH 3.2; and (B) acetonitrile. The gradient applied was: 0–2 min 20% B; 2–22 min 20–85% B; 22–25 min 85–100% B; 25–28 min 20% B; equilibration time 7 min; temperature 25 °C; flow rate 0.4 mL/min. In this case, the chromatograms were acquired at 224 nm. The linearity range of responses of Sb and FITC dissolved in CH₃OH was determined on five concentration levels from 0.001 µg/µL to 0.054 µg/µL.

The encapsulation efficiency was calculated by the following equation:

$$\text{Encapsulation efficiency \%} = \frac{\text{SLM (or FITC) encapsulated}}{\text{Total SLM (or FITC)}} \times 100$$

The recovery was estimated following the same procedure but without dialysis purification and was calculated by using the following equation:

$$\text{Recovery \%} = \frac{\text{SLM (or FITC) detected}}{\text{Total SLM (or FITC)}} \times 100$$

Storage stability studies

Stability studies on particle size, PDI, ζ -potential and encapsulation efficiency of SLM-NLCs as suspension at 4 °C and freeze-dried SLM-NLCs stored at room temperature were conducted at days 0, 10, 20 and 30 from the production by light scattering and HPLC analyses. Phase separation and turbidity were also investigated in the case of the suspensions, while the freeze-dried NLCs were evaluated for the appearance of the powder and for the redispersibility time.

Stability in Simulated Gastrointestinal Conditions

The NLCs samples were incubated in an equal volume of simulated gastric fluid (SGF) for 2 h at 37 °C and subsequently in simulated intestinal fluid (SIF) for further 2 h at 37 °C. SGF was prepared dissolving pepsin (0.32% w/v), 2 g of sodium chloride and 7 mL of HCl in 1 L of distilled water. The pH of the solution was adjusted to 2.0 with HCl 1 M. The composition of SIF was: lipase (0.04% w/v), bile salts (0.07% w/v), pancreatin (0.05% w/v) and calcium chloride 750 mM. The pH was adjusted to 7.0 with NaOH. The effect of SGF and SIM on physical properties of NLCs was evaluated by measuring particle size and PDI (Aditya et al., 2013).

In Vitro Release

In vitro release of SLM from NLCs was investigated using the dialysis bag method. Regenerated cellulose membranes with an MWCO of 3.5 kD were used to retain the NLCs and allow the diffusion of SLM into the release medium. Phosphate buffer saline (PBS pH 7.4), SGF without pepsin and enzyme-free SIF were taken as dissolution medium. Two millilitres of SLM-loaded NLCs were deposited into the dialysis membrane and placed in 200 mL of release medium at 37°C with stirring at 150 rpm. At definite time intervals up to 24 h, 1 mL was withdrawn and replaced with fresh dissolution medium. SLM released was quantified by HPLC.

For evaluating the kinetic and mechanism of drug release, Korsmeyer–Peppas model, Hixson-Crowell model, Higuchi model, and first- and zero-order mathematical models were used and the best-fitted model was selected based on high regression coefficient (R^2) value for the release data.

PAMPA Experiments

The permeation of free SLM and SLM-NLCs through artificial membranes was determined using the protocol previously reported (Bergonzi et al., 2014). The 96-well microtiter PVDF filter plate was impregnated with 10 μ L of a lecithin (1% w/v) and cholesterol (0.8% w/v) in 1,7-octadiene solution. Then, the donor plate was filled with 250 μ L of SLM solution or SLM-NLCs and placed upon the acceptor plate containing 250 μ L of 5% v/v DMSO in PBS. The plate assembly was placed into a sealed container to prevent evaporation and incubated at room temperature for 4 h. After the incubation, the samples were diluted with methanol, sonicated in an ultrasonic bath for 30 min and centrifuged for 10 min at 14000 rpm for HPLC analyses.

The permeability of SLM and the recovery value were calculated according to the equations reported in the literature (Wohnsland & Faller, 2001; Sugano et al., 2001):

$$P_e = C \times -\ln \left(1 - \frac{[\text{Compound}]_A}{[\text{Compound}]_{eq}} \right); \text{ where } C = \frac{V_D \times V_A}{(V_D + V_A) \times A \times t};$$

$$[\text{Compound}]_{eq} = \frac{[\text{Compound}]_D \times V_D + [\text{Compound}]_A \times V_A}{(V_D + V_A)}$$

$$\text{Recovery (\%)} = \frac{[\text{Compound}]_D \times V_D + [\text{Compound}]_A \times V_A}{([\text{Compound}]_{D_0} \times V_D)} \times 100$$

where P_e is the effective permeability (cm/s); A is the effective filter area (cm²); V_D and V_A are the volumes in the acceptor and the donor plates (mL), respectively; t is incubation time (s); $[\text{Compound}]_A$ and $[\text{Compound}]_D$ are the concentrations of SLM in the acceptor and the donor plates at time t , respectively; and $[\text{Compound}]_{D_0}$ is the concentration of SLM in the donor plate at time 0.

Caco-2 cell line

The colorectal adenocarcinoma cell line Caco-2 was purchased from American Tissue Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Rodano, Milan, Italy) with 20% fetal bovine serum (FBS) (Thermo Fisher Scientific, Rodano, Milan, Italy), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Rodano, Milan, Italy) in 5% CO₂ at 37 °C.

Cell Culture Experiments

An MTS assay was used to determine the cell viability after exposure to NLCs for 2 h, as previously described (Bigagli et al., 2017). The relative cell viability was expressed as a percentage of the untreated control group.

Transport Studies

For the permeation studies, Caco-2 cells were seeded into 12-well PET transwell plates (1.13 cm² growth surface area and pore size 0.4 µm, Greiner Bio-One, Milan, Italy) at a density of 2×10^4 cells/cm² and grown for 21 days to form a confluent monolayer. The integrity of the cellular barrier was assessed using Lucifer Yellow permeability test (Iacomino et al., 2013). Samples of media were collected from the basal compartment of each transwell plate and replaced with fresh HBSS at 0 (before sample addition), 30 and 60 min. The FITC concentration of these samples was determined by HPLC and adjusted to reflect the change in volume at each successive sampling point. LY measurements were also taken at the end of the experiment.

Uptake Studies

To identify the transepithelial transport mechanism, Caco-2 cells were pre-incubated with sodium azide (an ATP synthesis inhibitor, 1 µM), chlorpromazine (a clathrin blocker, 15 µM), and indomethacin (a caveolin-dependent endocytosis inhibitor, 25 µM) for 30 min followed by addition of NLCs 1:180 for 1 h, or maintained at 4°C during the NLCs exposure. At the end of the treatments, the amount of FITC was measured on cellular lysate by HPLC analysis. In parallel, Caco-2 cells, grown on histological slides, were treated in the same condition, fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 10 min and observed by fluorescence microscopy (Labophot-2 Nikon, Tokyo, Japan). Ten photomicrographs were randomly taken for each sample and fluorescence was measured using ImageJ 1.33 image analysis software (<http://rsb.info.nih.gov/ij>).

In vivo experiments

Animals

Male CD-1 mice (Envigo, Varese, Italy) weighing approximately 20 g at the beginning the experimental procedure were used. Animals were used in Ce.S.A.L (Centro Stabulazione Animali da Laboratorio, University of Florence, Italy) and used at least one week after their arrival. Twelve mice were housed per cage (size 26 x 41 cm) kept at 23.0 ± 1.0 °C with a 12 h light/dark cycle, with lights on at 7 a.m.; during acclimatization they were fed a standard laboratory diet and tap water ad libitum.

All animal's manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union Council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath & Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Modeling of metabolic disease

Disease Metabolic syndrome was induced feeding the animals with a high-fat diet (HFD; Research Diets, New Brunswick, NJ) for 6 weeks *ad libitum*. The model is consistent, with minor modification, to what was previously published (Reed et al., 2000; Srinivasan et al., 2005; Mali et al., 2014). HFD contains: 60% fat, 20% protein and 20% carbohydrate, as a percentage of total Kcal (Watanabe et al., 2012). Control animals were fed *ad libitum* for 6 weeks with a standard diet (24% protein, 58% carbohydrate, 18% fat; Envigo, Varese, Italy). As additive treatment to boost the hyperglycaemic component, a single intraperitoneal injection of streptozotocin (STZ) at a dose of 100 mg kg⁻¹ body weight in freshly prepared citrate buffer (0.1 M pH 4.0; Malekinejad et al., 2012) was done on day 15, two weeks after the beginning of animal feeding with the high fat or normal diet.

Toxicity and Irwin test

Toxicity was evaluated administering empty NLCs and SLM-loaded NLCs twice daily for 3 days. For the Irwin test, each mouse was individually placed in a transparent cage (26 × 41 cm), and 26 neurobehavioral or physiological parameters were systematically assessed according to Irwin, 1968. Behavioural, autonomic, and neurological manifestations produced by compound administration in rats were evaluated: motor displacement, motor reflexes, stereotypies, grooming, reaction to painful or environmental stimuli (analgesia, irritability), startle response, secretions, excretions, respiratory movements, skin colour and temperature, piloerection, exophthalmos, eyelid and corneal reflexes, muscle tone, ataxia, tremors, head twitches, jumps, convulsions, Straub tail, and other signs or symptoms. For postural reflexes (righting reflex) and other signs such as piloerection, exophthalmia (exaggerated protrusion of the eyeball), ataxia, tremors, and Straub tail, only presence or absence was recorded.

Skin colour was evaluated qualitatively (pale, red, or purple); other signs were evaluated semi-quantitatively, according to the observer's personal scale (0 to +4, -4 to 0, or -4 to +4). The terms sedation and excitation express the final interpretation of a group of signs: reduced motor activity, reduced startle response, eyelid ptosis, and reduced response to manual manipulation, for the former; and increased motor activity, increased startle response, increased response to manual manipulation, and exophthalmia, for the latter. Hyperactivity includes running, jumps, and attempts to escape from the container. Trained observers not informed about the specific treatment of each animal group carried out this test.

Treatments

Silymarin 38.13 mg kg⁻¹ suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich, Milan, Italy), SLM-NLCs-SA 38.13 mg kg⁻¹ and SLM-NLCs-CP 38.13 mg kg⁻¹ were administered twice daily *per os* for four weeks. Control animals were treated with the vehicle.

Collection of blood and analytical methods

Blood was collected weekly from the facial vein of the mice under light ether anaesthesia into eppendorf tubes containing heparin (20 µL, 25000 IU/5 mL). Samples were analyzed for glucose, triglycerides and total cholesterol levels using a Reflotron reflectance photometric analyzer (Reflotron, Roche Diagnostics and Vitros, Johnson & Johnson, Monza (MB), Italy).

Cold plate test

The animals were placed in a stainless steel box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Pain-related behaviour (licking of the hind paw) was observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s (Di Cesare Mannelli et al., 2013).

Statistical analysis

Results were expressed as means $\pm$ S.E.M. of 12 mice per group and the analysis of variance was performed by ANOVA test. A Bonferroni's significant difference procedure was used as a post hoc comparison. P values less than 0.05 were considered significant. Data were analyzed using the "Origin 9.1" software.

RESULTS AND DISCUSSION

NLCs Preparation and Characterization

The selection of the components for NLCs preparation is essential to obtain good stability of the formulation and high encapsulation efficiency of the extract. For this purpose, solubility studies of SLM in different liquid lipids (Table 1) have been performed. The selected excipients are approved and recognized as safe substance, as the purpose of this study is to produce formulations suitable for oral administration.

Liquid Lipid	SLM Solubility (mg/mL)
Borage oil	0.12 ± 0.01
Capryol 90	13.80 ± 0.14
Hemp oil	0.13 ± 0.01
Labrafac PG	0.77 ± 0.01
Labrafac WL 1349	0.72 ± 0.09
Labrafil M 1994	2.10 ± 0.15
Lauroglycol 90	10.00 ± 0.01
Oleic acid	-
Vitamin E	0.53 ± 0.15

Table 1. Solubility of silymarin (SLM) in different liquid lipids/oils.
Results are expressed as means $\pm$ standard deviation, n = 3.

As evidenced in Table 1, the solubility of SLM in the various analyzed liquid lipids is always higher than in water (0.26 mg/mL), except in the case of borage and hemp oil. The best result was obtained using Capryol 90 (13.80 mg/mL) and Lauroglycol (10.00 mg/mL).

Capryol 90 is a colourless liquid with HLB of 5, consisting of mono propylene glycol/esterified with caprylic acid and used for oral, topical, rectal and vaginal formulations. Lauroglycol 90 is an oil with HLB equal to 3, consisting of mono/di-esterified propylene glycol with lauric acid and used as an excipient for topical and oral pharmaceutical formulations.

The choice of lipids and surfactants used for NLCs formulation was performed by evaluating not only the solubility of SLM but also the HLB of oils and tensides, with the aim to obtain good encapsulation efficacy and high stability of the nanoparticles. Stearic acid, cetyl palmitate and Precirol ATO were tested as solid lipids, Capryol and Lauroglycol as liquid lipids. Among the surfactants, selected hydrophilic compounds were Tween 80 (HLB 15), Tween 20 (HLB 16.7) and brij 78 (HLB 15). These tensides were chosen based on the HLB value and solubilizing capacities.

Different ratios of solid and liquid lipids and tensides were evaluated. Some tested formulations showed good physical requires for oral administration, such as size and PDI, but they were not stable. Finally, the optimized NLCs formulations are reported in Table 2. Their compositions were Stearic acid/ brij 78 (1:2 w/w) plus Capryol 90 (2:8 w/w respect to solid lipid) and cetyl palmitate/brij 78 (1:3 w/w) plus Lauroglycol 90 (2:8 w/w respect to solid lipid). Brij 78 is a non-ionic tenside, derived from stearic acid covalently grafted with PEG 1000, which form a hydrophilic steric barrier around the NLCs and increase their stability. These selected formulations were able to load 40 mg of extract, with an EE% of 92.4% for SLM-NLCs-SA and 93.2% for SLM-NLCs-CP. The presence of SLM does not influence the stability of the NLCs: when SLM was loaded into the nanoparticles, the formulations showed almost the same physical parameters than empty NLCs.

TEM micrographs provided information on morphology and dimensions of nanoparticles: the NLCs had a spherical structure with a diameter like that obtained through DLS analyses (Figure 1).

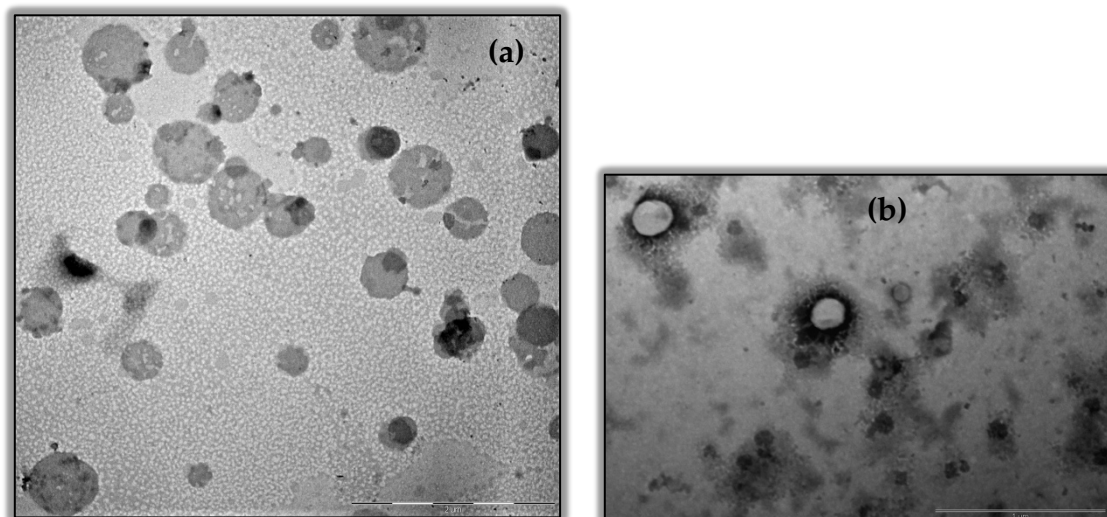


Figure 1. TEM images of SLM-NLCs-SA **(a)** and SLM-NLCs-CP **(b)**.

Scale bar 2 μm and 1 μm , respectively.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

FITC, a lipophilic fluorescent dye, was incorporated into NLCs as a probe to perform *in vitro* studies with Caco-2 cells and to elucidate trans-endothelial transport. Fluorescent NLCs were prepared as reported for empty NLCs, by adding FITC to the lipid phase (FITC-NLCs). Their physical and chemical parameters are shown in Table 2. All formulations are useful for oral administration. The high ζ -potential confirms their stability, as also supported by colloidal stability data.

Furthermore, the method applied for the preparation of the NLCs allows avoiding the degradation of the loaded extract or probe, as evidenced by the high recovery values (Table 2).

Sample	Size (nm)	PdI	ζ -potential (mV)	EE (%)	Recovery (%)
NLCs-SA	194.5 $\pm$ 3.7	0.24 $\pm$ 0.02	-36.0 $\pm$ 0.9	-	-
SLM-NLCs-SA	213.6 $\pm$ 16.0	0.17 $\pm$ 0.04	-31.6 $\pm$ 0.5	92.4 $\pm$ 4.1	94.8 $\pm$ 1.8
FITC-NLCs-SA	215.0 $\pm$ 0.3	0.24 $\pm$ 0.01	-37.4 $\pm$ 0.9	95.7 $\pm$ 1.2	98.5 $\pm$ 0.5
NLCs-CP	223.3 $\pm$ 2.4	0.26 $\pm$ 0.03	-29.5 $\pm$ 1.3	-	-
SLM-NLCs-CP	265.9 $\pm$ 13.4	0.24 $\pm$ 0.01	-34.5 $\pm$ 8.1	93.2 $\pm$ 4.6	96.2 $\pm$ 3.0
FITC-NLCs-CP	262.4 $\pm$ 4.2	0.27 $\pm$ 0.02	-33.4 $\pm$ 2.5	92.7 $\pm$ 2.3	98.8 $\pm$ 0.4

Table 2. Physical and chemical properties of empty NLCs, SLM-NLCs and FITC-NLCs. Results are expressed as means $\pm$ standard deviation, n = 3.

Then, the SLM-NLCs were freeze-dried in the presence of a cryoprotectant, both to prolong its physical and chemical stability over time, and to reduce microbial contamination. From the literature, different cryoprotectants, are reported, such as glucose, maltose, trehalose, sucrose, mannitol and lactose, from 5 to 20% w/v (Mehnert & Mader, 2001). Glucose (10% w/v) and mannitol (10% w/v) were tested in this work (Table 3).

Freeze-Dried Product	Particle Size (nm)	PdI	ζ -Potential (mV)	EE (%)	Yield (%)
SLM-NLCs-SA	383.6 $\pm$ 4.7	0.32 $\pm$ 0.03	-30.1 $\pm$ 0.8	64.1 $\pm$ 0.2	97.8 $\pm$ 0.1
SLM-NLCs-SA + Glucose	205.2 $\pm$ 2.7	0.16 $\pm$ 0.04	-36.7 $\pm$ 0.5	87.6 $\pm$ 4.9	98.9 $\pm$ 0.2
SLM-NLCs-SA + Mannitol	305.1 $\pm$ 9.5	0.24 $\pm$ 0.01	-32.6 $\pm$ 0.7	76.0 $\pm$ 3.1	99.2 $\pm$ 0.1
SLM-NLCs-CP	450.5 $\pm$ 6.8	0.41 $\pm$ 0.05	-32.4 $\pm$ 0.5	72.5 $\pm$ 0.1	98.4 $\pm$ 0.1
SLM-NLCs-CP + Glucose	269.1 $\pm$ 5.8	0.25 $\pm$ 0.03	-34.3 $\pm$ 0.2	88.5 $\pm$ 1.2	99.2 $\pm$ 0.1
SLM-NLCs-CP + Mannitol	288.4 $\pm$ 5.9	0.27 $\pm$ 0.02	-34.2 $\pm$ 0.1	87.5 $\pm$ 1.8	99.5 $\pm$ 0.2

Table 3. Physicochemical characterization and reaction yields of SLM-NLCs after freeze-drying process.

Results are expressed as means $\pm$ standard deviation, n = 3.

A sample for each SLM-NLCs formulation was lyophilized without cryoprotectant, while two other samples in the presence of glucose and mannitol, respectively. The dispersions were physically characterized by DLS after resuspension with water. The results (Table 3) highlighted a significant increase in size and PdI values in the absence of cryoprotectant. On the contrary, the lyophilization with glucose allowed preserving the stability of the system providing nanoparticles with almost identical dimensions and homogeneity those detected before the freeze-drying process. Finally, the nanoparticles lyophilized with mannitol revealed a certain increase regarding average diameter and inhomogeneity.

The results confirm the advantage of cryoprotectants in preventing the aggregation of nanoparticles during the lyophilization process. In particular, glucose allows obtaining a freeze-dried product easily re-dispersible, soft and voluminous. In fact, the cryoprotectants interact with the polar portions of surfactants, forming a kind of protective coating. They also reduce the crystallization processes, favouring the amorphous state of the frozen sample (Mehnert & Mader, 2001).

Chemical and Physical Storage Stability

After four weeks at 4°C, SLM-NLCs suspensions were chemically and physically stable. In the case of SLM-NLCs-SA, no changes in size and homogeneity occurred: dimensions were 200.3 ± 2.6 nm with PdI of 0.10 ± 0.03 . A slight increase of size for SLM-NLCs-CP was found (from 265.9 ± 13.4 nm to 300.2 ± 7.8 nm) but maintained properties suitable for oral administration.

The ζ -potential was monitored up to 30 days of storage, and no significant evolution was found, with a final value of -24.3 ± 0.7 mV for SLM-NLCs-SA and -33.1 ± 0.1 mV for SLM-NLCs-CP. Furthermore, EE% and recovery% did not dramatically decrease during storage (SLM-NLCs-SA: EE% 86.7 ± 2.4 , recovery% 89.3 ± 1.2 ; SLM-NLCs-CP: EE% 86.7 ± 2.4 , recovery% 92.1 ± 1.7). Thus, developed formulations are able to prevent the degradation of the incorporated compound. This represents an important result, considering that, in many cases, chemical and physical instabilities were observed when NLCs were stored as aqueous suspension due to lipid crystallization, polymorphic transformations, aggregation phenomena and hydrolysis processes.

An analogous study was performed on freeze-dried NLCs. Both products maintained almost the same physical characteristics as before freeze-drying (size 216.7 ± 4.7 nm and -35.2 ± 1.3 mV, in the case of SLM-NLCs-SA; size 275.6 ± 2.1 nm and -33.8 ± 0.5 mV, in the case of SLM-NLCs-CP) but there was a small decrease in EE% (79.8 ± 2.5 for SLM-NLCs-SA and 75.6 ± 2.5 for SLM-NLCs-CP). However, the recovery% for both formulations resulted higher than 80%.

Stability in Simulated Gastrointestinal Fluids

NLCs have been formulated not only for increasing the solubility of the SLM but also to protect the active components from degradation during gastrointestinal transit. The integrity of the nanoparticles can represent an important prerequisite to reach the sites of absorption in the intestine and obtain increased uptake at the intestinal epithelium itself (T. Wang et al., 2017). For this purpose, the physical stability of the formulations in simulated gastrointestinal conditions was determined by subjecting them to simulated gastric digestion at pH 2.0 in the presence of pepsin, followed by simulated intestinal digestion in the presence of the pancreatin-lipase-bile salts mixture.

After 2 h of incubation in SGF followed by 2 h of incubation in SIF, NLCs resulted stable and no aggregation or degradation phenomena occurred (Table 4). This stability in acidic and intestinal conditions may be due to the effect of steric stabilization of non-ionic surfactant, brij 78, which decreases the flocculation and coalescence phenomena with its molecular structure. In addition, this stability can also be conferred by the properties of lipids, able to protect encapsulated SLM from enzymatic degradation and unfavourable pH conditions (Narang et al., 2015).

Sample	SGF	SIF
NLCs-SA	258.4 ± 3.9	205.2 ± 2.8
NLCs-CP	231.1 ± 3.3	233.5 ± 1.7

Table 4. Physical stability of NLCs-SA and NLCs-CP in simulated gastrointestinal fluids. Results are expressed as means ± standard deviation of three experiments.

In Vitro Release

In vitro release studies were performed in different pH media without enzymes: PBS (pH 7.4), SGF (pH 2) and SIF (pH 7). The studies have been conducted to evaluate if and how different pH conditions can influence the release of SLM from the NLCs. The *in vitro* release profiles are shown in Figure 2. In physiological pH conditions (PBS, pH 7.4), the absence of the burst effect is evident: the release of SLM is not immediate but gradual, up to a maximum percentage of 66.3% for SLM-NLCs-SA and 53.9% for SLM-NLCs-CP in the 24 h. In the gastric environment during the first 2 h, corresponding to transit through the stomach, only the 9.6% of the SLM was released from SLM-NLCs-SA and 5.8% from SLM-NLCs-CP. Thus, the developed NLCs remain intact in SGF, according to the previous data of the stability study, they prevent degradation of the encapsulated extract and can be promising carriers for the transport of a large amount of SLM into the intestine.

In the simulated intestinal environment, a controlled release of SLM with a maximum of 15.4% released within 24 h from SLM-NLCs-SA and 14.8% from SLM-NLCs-CP was observed. These results suggest that, following the gastrointestinal tract transit, most of SLM remains encapsulated in NLCs, allowing greater intestinal absorption.

The low release obtained in simulated gastro-intestinal conditions suggests that the formulations protect SLM from degradation and guarantee a slow release of the extract due to the lipid matrix. NLCs demonstrated promising potential to prevent burst release and degradation of SLM which remained entrapped into nanoparticles and could be absorbed and reach the target site after oral administration (Luo et al., 2015).

The absence of the burst effect can be justified by the low percentage of liquid lipid which does not lead to the formation of an outer shell containing high concentrations of SLM, but the oil is distributed randomly in the solid lipid matrix (Jia et al., 2009). Such a prolonged release can allow increased absorption of the drug, maintaining a constant concentration in the plasma.

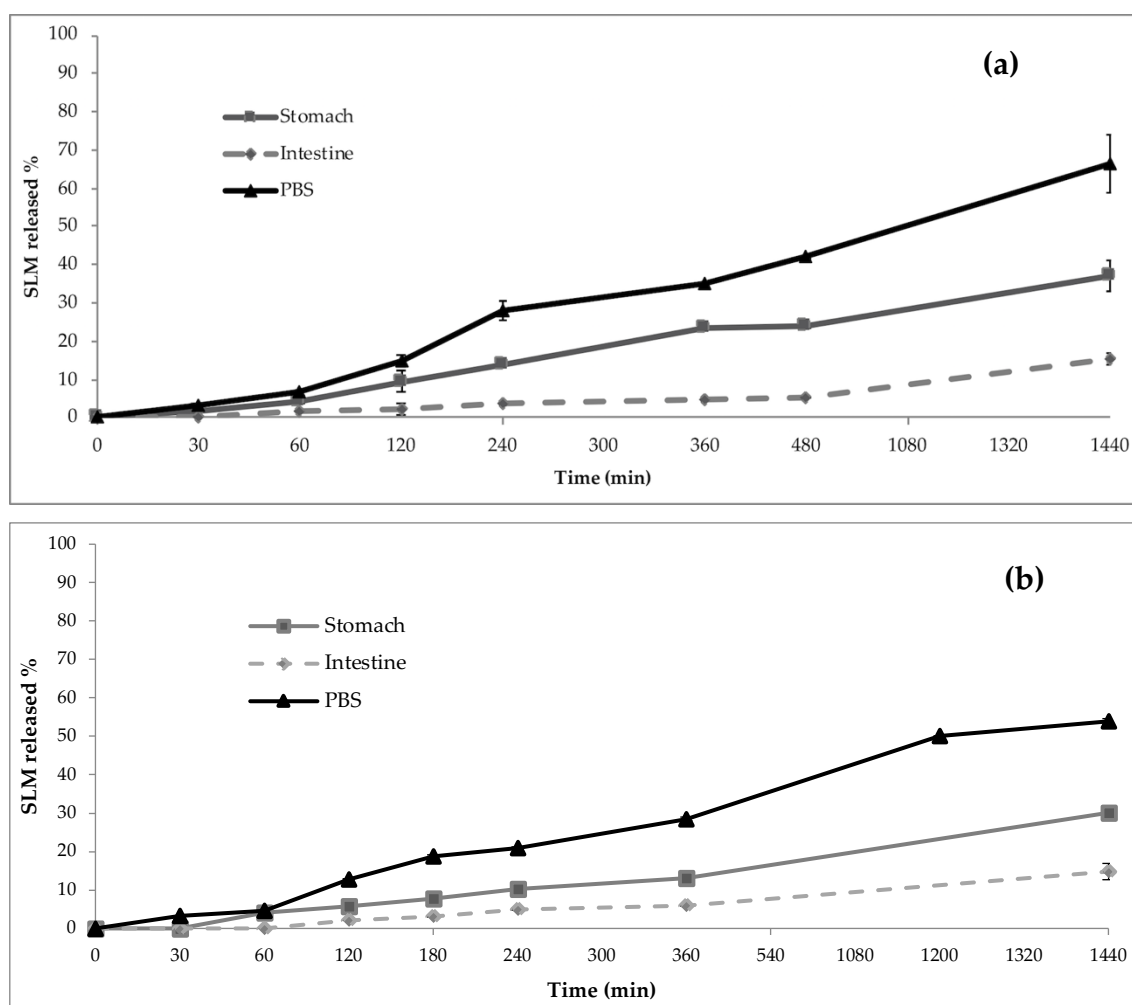


Figure 2. *In vitro* release studies of SLM from NLCs-SA (a) and NLCs-CP (b) in PBS pH 7.4 (black lines) and in simulated gastrointestinal fluids (dark grey line and light grey dotted line). Results are expressed as means $\pm$ standard deviation, $n = 3$.

Different theoretical models were considered to examine the kinetic of release. SLM release mechanism in PBS was defined by fitting SLM release data during 24 h to various kinetics models. By comparing the regression coefficient values, the Higuchi model was shown to be the most adequate to describe the kinetics of NLCs. Thus, the diffusion process controlled the release mechanism. Furthermore, analyzed according to Korsmeyer–Peppas model, the release exponent n was >0.89 , and it confirms that the release follows a super case II drug-transport mechanism and can be inferred that it is related to a non-Fickian diffusion process.

PAMPA Studies

The PAMPA is an *in vitro* method used to estimate transcellular passive permeability.

The effective permeability (P_e) value after 4 h of incubation was $12.1 \pm 1.4 \times 10^{-6}$ for free SLM, $132 \pm 11 \times 10^{-6}$ in the case of extract loaded into NLCs-SA and $129 \pm 2 \times 10^{-6}$ for SLM encapsulated into NLCs-CP. These results clearly indicated that passive permeation of SLM was improved considerably with respect to the aqueous solution (about 11-fold higher) when it is formulated. The recovery was 100% for the SLM-NLCs and 95% for the saturated aqueous solution of SLM. A high recovery value, above 80% is a proof of an acceptable permeation prediction. Moreover, this result suggests that there was neither relevant extract membrane retention nor binding to the surface of the filter. The developed formulations represent a successful attempt to ameliorate passive permeation of SLM.

Caco-2 Cell Culture Experiments

Permeation studies were performed using a cell-based model to complete *in vitro* characterization of NLCs and understand oral absorption mechanisms. For this aim, Caco-2 cells are considered the most predictive *in vitro* model to estimate not only passive intestinal diffusion, as previously described for PAMPA but also active transport processes, paracellular permeability and active efflux.

The cytotoxicity of the developed NLCs was first investigated by MTS test with the aim to select the most appropriate concentration for transport studies. Both formulations exerted quite low cytotoxicity (about 30%) diluted 1:180. This is a good result considering that NLCs undergo a dilution from 1:200 to 1:1000 when they are orally administered.

Thus, the transport studies were conducted by using this dilution and 1 h as exposition time. P_{app} values are reported in Figure 3; it can be observed that the permeation of FITC, a lipophilic probe not able to cross the Caco-2 monolayer, was improved when formulated into NLCs, reaching an apparent permeability of 102.4×10^{-6} cm/s in the case of NLCs-SA and 90.7×10^{-6} cm/s in the case of NLCs-CP, after 30 minutes of incubation.

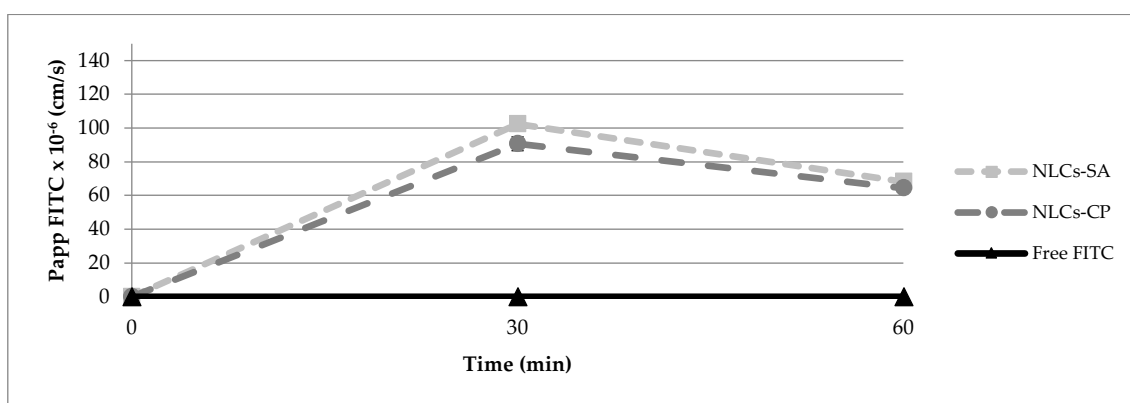


Figure 3. Apparent permeability coefficient (P_{app}) of free FITC and FITC loaded into NLCs (FITC-NLCs) across the Caco-2 monolayer. Results are expressed as means $\pm$ standard deviation, $n = 3$.

To investigate the possible pathways of FITC-NLCs internalization in Caco-2 cells, their uptake was followed in different energetic conditions and in the presence of endocytosis inhibitors.

Figure 4 showed that the internalization of the FITC-NLCs was significantly reduced when incubated at different energetic conditions (4°C and ATP-depleted condition). The experimental results indicated, in fact, that the cellular uptake of FITC-NLCs-SA at 4 °C was only about 37% and about 33% for FITC-NLCs-CP of that at 37°C and sodium azide (an energy inhibitor) was also able to reduce, although to a lesser extent, the cellular uptake. At low temperatures, higher rigidity and lower permeability of lipid bilayer have been demonstrated and could explain this difference (Patrick et al., 2016).

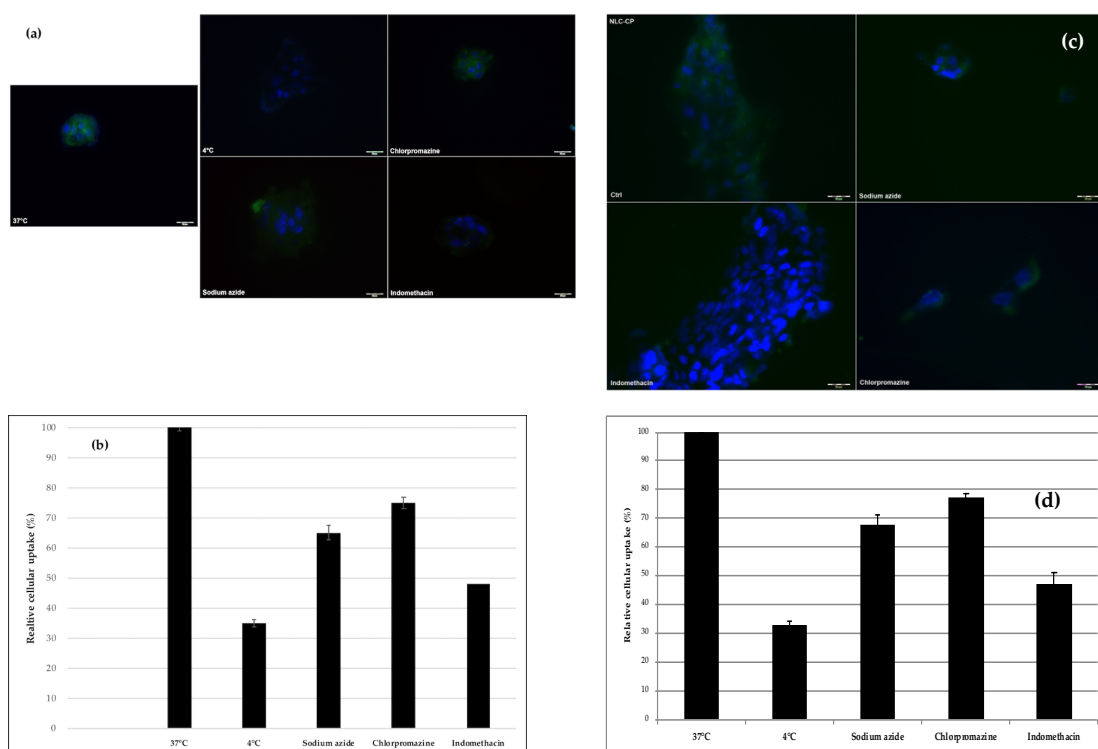


Figure 4. (a) and (c) Cellular uptake of FITC-NLCs (green staining), in the presence of endocytic inhibitors. Nuclei stained with DAPI. Final magnification 400×, scale bar 20 μm.

(b) and (d) Percentage of intracellular FITC in Caco-2 cells after 1 h of exposure to FITC-NLCs at 37 °C (100%) or in the presence of endocytic inhibitors.

Results are expressed as means ± standard deviation, n = 3.

The dependency of cellular uptake on energy indicates that the internalization possibly occurs by endocytosis. To further illustrate about the internalization pathway involved, Caco-2 cells were pretreated with inhibitors: chlorpromazine blocks clathrin-dependent endocytosis and indomethacin inhibits caveolin-dependent endocytosis. The cellular uptake of FITC-NLCs seems to be a rather complex process not dependent solely on a single mechanism. Our results indicated that they are incorporated into the intestinal epithelium mainly via the caveolin-dependent endocytic pathway, whereas the endocytosis mediated by clathrin was less involved in the uptake, reducing it by about 20% (Figure 4).

In vivo experiments*Toxicity*

Empty NLCs and SLM-loaded NLCs were administered twice daily for 3 days for the evaluation of toxicity and the results are reported in the Table 5 and Table 6.

	NLCs-SA	SLM-NLCs-SA	Limits
Volume/Dose	400 µL	38.13 mg/kg	
Behaviour			
<i>Spontaneous activity</i>	4	4	4 - 0
<i>Passivity</i>	0	0	0 - 4
<i>Cleaning</i>	0	0	4 - 0
<i>Curiosity</i>	4	4	4 - 0
<i>Reactivity</i>	4	4	4 - 0
<i>Vocalization</i>	0	0	0 - 4
S.N.C. excitement			
<i>Straub tail</i>	0	0	0 - 4
<i>Tremors</i>	0	0	0 - 4
<i>Convulsions</i>	0	0	4 - 0
Movement			
<i>Ataxia</i>	0	0	0 - 4
<i>Stereotopies</i>	0	0	0 - 4
<i>Straightening reflex</i>	4	4	4 - 0
Muscular tone			
<i>Physical strenght</i>	4	4	4 - 0
Reflexes			
<i>Palpebral reflex</i>	4	4	4 - 0
Autonomic signes			
<i>Piloerection</i>	0	0	0 - 4
<i>Exolpthalmos</i>	0	0	0 - 4
<i>Cyanosis</i>	0	0	0 - 4
<i>Flush</i>	0	0	0 - 4
<i>Pallor</i>	0	0	0 - 4
<i>Palpebral opening</i>	4	4	4 - 0
<i>Salivation</i>	0	0	0 - 4
<i>Lacrimation</i>	0	0	0 - 4
<i>Hypo-hyperthermia</i>	0	0	-4/+4
<i>Writhing</i>	0	0	0 - 4
Toxicity			
<i>Immediate death</i>	0	0	0 - 4
<i>Delayed death (48 h)</i>	0	0	0 - 4

Table 5. Behavioural, autonomic and neurological parameters of mice evaluated by the Irwin test, after treatment with empty and SLM-loaded NLCs-SA.

	NLCs-CP	SLM-NLCs-CP	Limits
Volume/Dose	400 µL	38.13 mg/kg	
Behaviour			
<i>Spontaneous activity</i>	4	4	4 - 0
<i>Passivity</i>	0	0	0 - 4
<i>Cleaning</i>	0	0	4 - 0
<i>Curiosity</i>	4	4	4 - 0
<i>Reactivity</i>	4	4	4 - 0
<i>Vocalization</i>	0	0	0 - 4
S.N.C. excitement			
<i>Straub tail</i>	0	0	0 - 4
<i>Tremors</i>	0	0	0 - 4
<i>Convulsions</i>	0	0	4 - 0
Movement			
<i>Ataxia</i>	0	0	0 - 4
<i>Stereotopies</i>	0	0	0 - 4
<i>Straightening reflex</i>	4	4	4 - 0
Muscular tone			
<i>Physical strenght</i>	4	4	4 - 0
Reflexes			
<i>Palpebral reflex</i>	4	4	4 - 0
Autonomic signes			
<i>Piloerection</i>	0	0	0 - 4
<i>Exolpthalmos</i>	0	0	0 - 4
<i>Cyanosis</i>	0	0	0 - 4
<i>Flush</i>	0	0	0 - 4
<i>Pallor</i>	0	0	0 - 4
<i>Palpebral opening</i>	4	4	4 - 0
<i>Salivation</i>	0	0	0 - 4
<i>Lacrimation</i>	0	0	0 - 4
<i>Hypo-hyperthermia</i>	0	0	-4/+4
<i>Writhing</i>	0	0	0 - 4
Toxicity			
<i>Immediate death</i>	0	0	0 - 4
<i>Delayed death (48 h)</i>	0	0	0 - 4

Table 6. Behavioural, autonomic and neurological parameters of mice evaluated by the Irwin test, after treatment with empty and SLM-loaded NLCs-CP.

Behavioural, autonomic and neurological parameters were evaluated giving an arbitrary score (from 0 to ± 4) to assess the toxicity of the formulations by the Irwin test. As evidenced in Table 5 and Table 6, no toxicity was recorded after the repeated administration of SLM-NLCs-SA and SLM-NLCs-CP, both at 38.13 mg/kg, or empty NLCs.

Hematic metabolic parameters

Animals were fed *ad libitum* from week -2 to week 4 with a high-fat diet (60% fat) or a standard diet (10% fat). On week 1, diabetes was induced by a single intraperitoneal injection of STZ (100 mg kg⁻¹) and concomitantly treatments with SLM, SLM-NLCs-SA and SLM-NLCs-CP started. Blood glucose was monitored in each group from week 1 to week 4 as reported in Table 7. STZ treatment caused a rise in blood glucose ($P < 0.01$) in comparison to that in the control group (normal diet + vehicle). Twice daily treatment with SLM-NLCs-SA (38.13 mg kg⁻¹) reduced blood glucose level ($P < 0.01$) starting from the first week of administration until the end of the experiment (week 4). SLM-NLCs-CP reached the statistical significance only on week 1. SLM was ineffective (Table 7).

Treatments	Glucose (mg/dL)			
	week 1	week 2	week 3	week 4
Normal diet + Vehicle	132.5 $\pm$ 0.5	96.0 $\pm$ 12.0	128.0 $\pm$ 1.0	122.3 $\pm$ 22.6
HFD + STZ + Vehicle	555.3 $\pm$ 37.2**	462.0 $\pm$ 21.7**	561.0 $\pm$ 27.6**	480.3 $\pm$ 31.9**
HFD + STZ + SLM	520.7 $\pm$ 26.0	371.7 $\pm$ 21.7	563.3 $\pm$ 13.4	443.5 $\pm$ 21.2
HFD + STZ + SLM-NLCs-SA	197.3 $\pm$ 12.8^^	189.0 $\pm$ 50.2^^	288.1 $\pm$ 44.4^^	149.2 $\pm$ 19.7^^
HFD + STZ + SLM-NLCs-CP	302.0 $\pm$ 39.6^	367.0 $\pm$ 65.5	506.6 $\pm$ 63.2	414.3 $\pm$ 30.4

Table 7. Blood glucose levels from week 1 to week 4.

Silymarin (38.13 mg/kg) was suspended in 1% CMC and *per os* administered twice daily. SLM-NLCs-SA (38.13 mg/kg) were *per os* administered twice daily, in the morning and in the evening. SLM-NLCs-CP (38.13 mg/kg) were *per os* administered twice daily, in the morning and in the evening.

**P < 0.01 vs normal diet + Vehicle; ^P < 0.05 and ^^P < 0.01 vs HFD + STZ + Vehicle.

Each value represents the mean $\pm$ s.e.m. of 12 mice per group

In Table 8, blood levels of triglycerides and cholesterol are reported. Measurements were performed on week 2 and 4, starting from the beginning of SLM and SLM-NLCs administration. STZ increased triglycerides levels ($P < 0.01$) in comparison to the control group. SLM-NLCs-SA reduced triglycerides both on week 2 and 4 ($P < 0.01$) while SLM-NLCs-CP treatment only on week 2 ($P < 0.05$). Cholesterol was not modified by STZ, SLM and SLM-NLCs administration in comparison to the control animals (Table 8).

Treatments	Triglyceride (mg/dL)		Total cholesterol (mg/dL)	
	week 2	week 4	week 2	week 4
Normal diet + Vehicle	143.0 $\pm$ 37.7	180.7 $\pm$ 20.1	106.0 $\pm$ 6.6	133.7 $\pm$ 15.3
HFD + STZ + Vehicle	502.0 $\pm$ 84.9**	506.8 $\pm$ 93.3**	119.8 $\pm$ 6.9	169.5 $\pm$ 14.2
HFD + STZ + SLM	368.3 $\pm$ 126.5	417.3 $\pm$ 95.4	110.7 $\pm$ 5.2	157.8 $\pm$ 12.0
HFD + STZ + SLM-NLCs-SA	214.0 $\pm$ 13.3^^	168.3 $\pm$ 10.2^^	124.8 $\pm$ 3.2	133.8 $\pm$ 11.3
HFD + STZ + SLM-NLCs-CP	232.5 $\pm$ 40.7^	408.0 $\pm$ 62.1	123.8 $\pm$ 8.4	163.0 $\pm$ 6.0

Table 8. Blood levels of triglycerides and cholesterol measured on week 2 and 4.

Silymarin (38.13 mg/kg) was suspended in 1% CMC and *per os* administered twice daily. SLM-NLCs-SA (38.13 mg/kg) was *per os* administered twice daily, in the morning and in the evening. SLM-NLCs-CP (38.13 mg/kg) was *per os* administered twice daily, in the morning and in the evening. ** $P < 0.01$ vs Normal diet + Vehicle; ^ $P < 0.05$ and ^^ $P < 0.01$ vs HFD + STZ + Vehicle.

Each value represents the mean $\pm$ s.e.m. of 12 mice per group.

Behavioural measurements

On week 4, the Cold plate test was performed to assess the pain relief effect of SLM and SLM-NLCs on STZ-induced neuropathy (Table 9). Silymarin (38.13 mg/kg) was suspended in 1% CMC and *per os* administered twice daily. SLM-NLCs-SA (38.13 mg/kg) was *per os* administered twice daily, in the morning and in the evening. SLM-NLCs-CP (38.13 mg/kg) was *per os* administered twice daily, in the morning and in the evening.

The HFD + STZ group showed a significantly lowered nociceptive threshold in comparison to the control animals. Daily treatments with SLM, SLM-NLCs-SA and SLM-NLCs-CP significantly increased the licking latency of mice, in particular the compound SLM-NLCs-CP showed the best anti-hyperalgesic effect.

Cold plate test	
Licking latency (s)	
Treatments	week 4
Normal diet + Vehicle	18.4 ± 0.2
HFD + STZ + Vehicle	10.5 ± 0.6**
HFD + STZ + SLM	14.8 ± 0.5^^
HFD + STZ + SLM-NLCs-SA	15.0 ± 0.3^^
HFD + STZ + SLM-NLCs-CP	17.4 ± 0.4^^

Table 9. Cold plate test.

Pain-related behaviour (i.e. lifting and licking of the hind paw) were observed and the time (seconds) of the first sign was recorded. **P < 0.01 vs normal diet + vehicle; ^^P < 0.01 vs HFD + stz + vehicle.

Each value represents the mean ± s.e.m. of 12 mice per group.

CONCLUSIONS

This study confirmed that the developed NLCs represent promising delivery systems for enhancing the solubility and the intestinal permeability of SLM.

Two different NLCs formulations were optimized: one of them was made of stearic acid, as solid lipid, Capryol 90 as liquid lipid, and brij 78 as non-ionic surfactant; the ingredients of the other formulation were cetyl palmitate (solid lipid), Lauroglycol 90 (liquid lipid) and brij 78. Based on the obtained results of physical and chemical characterizations, developed formulations could be orally administered. The systems showed excellent chemical and physical stability during storage at 4°C and after incubation into simulated gastrointestinal environment. *In vitro* release indicated that NLCs might prevent burst release and gastric degradation of SLM. Moreover, the slow release of SLM suggests homogeneous entrapment of the compound throughout the lipid matrix.

After demonstrating were successful in enhancing the passive permeation of SLM by PAMPA, Caco-2 cells were selected to form simulative intestinal epithelial cell monolayers to study the permeation and the related transport mechanisms. The results indicated that the formulations were successful in enhancing the permeation of SLM and that the transport of the NLCs across the cell monolayer is energy-dependent and mediated by clathrin- and caveolae (or lipid raft)-related routes.

In order to evaluate the safety of the NLCs and to choose the best formulation to enhance the pharmacological potential of SLM, *in vivo* experiments were performed in an STZ-induced diabetic mouse model.

These studies indicated that the treatment with SLM-NLC-SA exhibited a significant down-regulation of blood glucose levels compared to the groups treated with free SLM by the first week of the study period. Besides, SLM-NLCs-SA reduce triglycerides levels significantly in comparison to free SLM. Furthermore, SLM-NLCs-SA and SLM-NLCs-CP showed a significant anti-hyperalgesic effect on STZ-induced neuropathy.

Based on these promising results, future studies will be aimed to perform histopathological investigations, to the evaluation of others blood parameters and to confirm and better clarify why SLM-NLCs-SA resulted better than SLM-NLCs-CP to reduce glucose and triglycerides blood levels in a diabetic mouse model.

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Nanostructured Lipid Carriers as Promising Delivery Systems for Plant Extracts: The Case of Silymarin

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SOLID LIPID NANOPARTICLES FOR DELIVERY OF ANDROGRAPHOLIDE ACROSS THE BLOOD-BRAIN BARRIER: IN VITRO AND IN VIVO EVALUATION

INTRODUCTION

Andrographolide (AG) is the major diterpenoid in the traditional Asian medicinal plant *Andrographis paniculata* (AP, Burm. f.) Wall. ex Nees (Acanthaceae) and it has received increasing attention due to its various pharmacologic activities as it is hepatoprotective, antiviral, antibacterial, as well as protective against oxidative stress-mediated neurotoxicity, inflammation-mediated neurodegeneration, and cerebral ischemia (Jayakumar et al., 2013; Yen et al., 2013). Recently, Serrano et al., 2014 showed that AG protects against damage induced by amyloid- β oligomers *in vitro*, lowers amyloid- β levels and tau phosphorylation in mice, modulates the formation of amyloid plaques, and recovers spatial memory functions in Alzheimer disease transgenic mouse model.

In vitro and *in vivo* studies proved that AG modulates complex oxidative stress-related pathways involved in stroke pathogenesis in primary cerebral endothelial cells and it provides positive protection against ischemic stroke (Yen et al., 2016). *In vitro* studies also demonstrated that AG inhibits the proliferation of human glioblastoma cells (Y. C. Li et al., 2012). Furthermore, recent studies suggested that it possesses anti-tumour activity, with various mechanisms being involved. The high lipid solubility of AG permits penetration of the blood-brain barrier (BBB) on the one hand, but on the other reduces its bioavailability due to poor water solubility and high chemical and metabolic instability. Indeed, these factors are the greatest limitations with regard to the development of new formulations for clinical use (M. Wang et al., 2009).

Several approaches have been applied to overcome these obstacles. Xie et al., 2016 developed AG nanocrystals to improve solubility and evaluated the impact of different stabilizers and matrix formers on their redispersibility. Chellampillai & Pawar, 2011 demonstrated that pH-sensitive nanoparticles significantly improve the dissolution rate and bioavailability of AG after oral administration in male Wistar albino rats. Several nanosystems have been proposed to enhance the anti-inflammatory effect and hepatoprotective properties of AG in recent years. In particular, toward this aim, Roy et al., 2012 developed heparin functionalized poly(D-L-lactide-co-glycolic acid) nanoparticles able to alleviate paracetamol hepatotoxicity in mice and cationic modified poly(D-L-lactide-co-glycolic acid) nanoparticles to increase the solubility, the hepatic residence and the cytokine regulation of AG in hepatotoxicity status (Roy et al., 2014). Das et al. demonstrated that poly (D-L-lactide-co-glycolic acid) nanocapsules reduced arsenic-induced liver damage in mice compared to free AG (Das et al., 2015). Furthermore, just this year, Qiao et al., 2017 proved that amorphous AG nanosuspensions are effective at enhancing oral bioavailability and biological efficacy, and Mishra et al., 2017 developed a multi-layered nanoemulsion and demonstrated improved hepatoprotection and absorption of AG when orally delivered. Other recently published studies have shown that the anti-leishmanial activity and anticancer efficacy of AG were considerably ameliorated when it is encapsulated in poly(D-L-lactide-co-glycolic acid) nanoparticles (Mondal et al., 2013). In addition, two attempts to incorporate AG into solid lipid nanoparticles (SLNs) have been proposed: Yang et al., 2013 carried out *in vitro* and *in vivo* studies to prove that nanoparticles enhance the antihyperlipidemic property of AG, while Parveen et al., 2014 evaluated the antitumor activity of AG and obtained better results with SLNs compared to the free-molecule. PLGA-nanoparticulation of AG enhanced its anti-cancer properties three fold and the chitosan coating of nanoparticles further accentuated cellular localization and increased cellular toxicity and apoptosis in MCF-7 cells (Roy et al., 2013).

The challenges regarding poor pharmacokinetic properties are even more evident for drugs directed toward the CNS due to the presence of the BBB (Abbott et al., 2010; Y. Chen & Liu, 2012). For this reason, various strategies have been developed to increase the bioavailability of neurotherapeutics: recent developments of nano-sized delivery systems seem to be the solution to this great problem (H. L. Wong, Wu, & Bendayan, 2012). These systems provide sufficient drug permeability into the brain and can be functionalized and interact with specific receptors of the BBB, favouring the crossing over of active molecules (Blasi et al., 2007).

In this context, we evaluated the use of solid lipid nanoparticles (SLNs) to deliver AG through the CNS and ameliorate its biopharmaceutical characteristics.

Essential ingredients for SLNs preparation include solid lipids at room temperature and at body temperature, surfactant/s, and water; the lipid matrix is made of biocompatible and biodegradable materials, which decrease the risk of acute or chronic toxicity. SLNs present many advantages such as long-term stability, increased bioavailability of encapsulated active ingredient, the possibility to obtain a controlled or targeted release, versatility in encapsulating both lipophilic or hydrophilic drugs, and high efficiency of encapsulation (Wissing et al., 2004). SLNs are also potential drug delivery systems for brain targeting (Dal Magro et al., 2017) as their small size prolongs circulation time in the blood and decreases the burst effect (Garcia-Garcia et al., 2005; Kaur et al., 2008). Continuing the studies on the development of drug delivery systems able to cross the BBB (Bergonzi et al., 2016; Grossi et al., 2017), the present study includes the incorporation of AG into SLNs. The nanoparticles were prepared by the emulsion/evaporation/solidifying method (J. Q. Zhang et al., 2007) using Brij 78, a non-ionic surfactant employed to obtain “stealth” and “long circulating” nanoparticles. This surfactant protects nanoparticles from interaction with plasma components, prolongs their half-life in blood circulation, and increases drug permeability by fluidization of cell membranes (Kaur et al., 2008).

SLNs were characterized in terms of average diameter, polydispersity, ζ -potential, morphology, and physical and chemical stability; *in vitro* tests were also performed.

The ability of nanoparticles to increase the permeability of AG was evaluated by Parallel Artificial Membrane Permeability Assay (PAMPA) (Di et al., 2003) and hCMEC/D3 cells, as a well-established *in vitro* BBB model (Eigenmann et al., 2013). Finally, fluorescent SLNs were developed to study *in vivo* distribution and fate after intravenous administration in healthy rats and to evaluate the ability of the delivery system to cross the BBB and reach brain tissues.

MATERIALS

Compritol 888 ATO, a mixture of mono-, di- and triglycerides of behenic acid, was a gift of Gattefossé (Saint Priest, France). Andrographolide, Brij 78 (Polyethylene glycol octadecyl ether), Fluorescein isothiocyanate (FITC), Human Serum Albumin (HSA), Caffeine, Piroxicam, Progesterone and Phosphate Buffered Saline (PBS 0.01 M) powder (29 mM NaCl, 2.5 mM KCl, 7.4 mM Na₂HPO₄·7H₂O, 1.3mM KH₂PO₄) pH 7.4 were from Sigma Aldrich (Milan, Italy). 96-well Multi-Screen PAMPA filter plates (pore size 0.45 µm) were purchased from Millipore Corporation (Tullagreen, Carrigtwohill, County Cork, Ireland). Porcine polar brain lipid was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All the solvents HPLC and analytical grade were from Sigma Aldrich (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA) and phosphotungstic acid (PTA) was purchased from Electron Microscopy Sciences (Hatfield, USA).

METHODS

Preparation of SLNs

Empty SLNs were prepared by the emulsion/evaporation/solidifying method (J. Q. Zhang et al., 2007). Compritol 888 ATO was dissolved in 5 mL of acetone under magnetic stirring and heated at $50^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Brij 78 was dissolved in 30 mL of water at $75^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The organic phase was added dropwise to the aqueous phase under fast magnetic stirring to obtain an emulsion that was concentrated to 7 mL to induce the evaporation of the organic solvent. The emulsion was added quickly to 7 mL of cold distilled water in an ice bath under fast magnetic stirring to obtain solidified nanoparticles. The colloidal dispersion was frozen and freeze-dried for one night.

AG-loaded SLNs (AG-SLNs) or FITC-loaded SLNs (FITC-SLNs) were prepared using the same method, adding AG or FITC ($\lambda_{\text{ex}}=492\text{ nm}$, $\lambda_{\text{em}}=518\text{ nm}$, green) to the lipid phase.

Different percentages of the drug were loaded to obtain AG-SLNs: 2.5%, 5%, and 10% (w/w respect to the amount of lipid and surfactant). Fluorescent SLNs contained 5% of FITC (3.67 mM).

Characterization of SLNs in terms of size, polydispersity index and ζ -potential

Particle size was measured by Light Scattering, Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25°C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_{average}) and the particle size distribution (polydispersity index, PDI) using ALV-60X0 software V.3.X provided by Malvern. ζ -potential was measured using the same instrument and was calculated from the electrophoretic mobility. For all samples, opportunely diluted with distilled water, an average of three measurements at stationary level was taken. The temperature was kept constant at 25 °C by a Haake temperature controller.

Morphological characterization of nanoparticles

SLNs were analyzed by transmission electron microscopy (TEM). The opportunely diluted aqueous dispersion was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film, which was stained with a phosphotungstic acid solution (1% w/v) in sterile water. Samples were dried for 3 min and were then examined under a JEOL 1010 electron microscope (Tokyo, Japan) and photographed at an accelerating voltage of 64 kV.

Differential scanning calorimetry (DSC)

DSC was carried out using a Mettler TA4000 apparatus equipped with a DSC 25 cell. Samples (about 5-10 mg) were accurately weighed (Mettler M3 Microbalance) directly in pierced aluminium pans and scanned between 30 and 250°C at a heating rate of 10 K/min under static air. DSC thermograms of pure AG, Compritol, brij 78 and AG-SLNs were compared.

Determination of encapsulation efficiency and loading capacity

The amount of AG or FITC entrapped in SLNs was calculated using the indirect method (Kaur et al., 2008). SLNs were purified from the free drug by ultracentrifugation for 60 min at $11330 \times g$ and $4\text{ }^{\circ}\text{C}$. Encapsulation efficiency (EE%) and loading capacity (LC%) were calculated considering the difference between the total amount of drug loaded and the amount of AG or FITC determined in the supernatants obtained after purification of the SLNs, using the following equations:

$$EE\% = \frac{(Total\ Drug - Free\ Drug)}{Total\ Drug} \cdot 100$$

$$LC\% = \frac{(Total\ Drug - Free\ Drug)}{Weight\ of\ SLNs} \cdot 100$$

The content of AG or FITC was quantified by HPLC-DAD or HPLC-FLD analysis, respectively.

HPLC-DAD and HPLC-FLD methods

Quali-quantitative determinations were carried out using an HP 1200 liquid chromatograph equipped with a DAD and FLD detector. A 150 mm x 4.6 mm i.d., 5 μm Zorbax Eclipse XDB, RP18 column (Agilent Technologies, Santa Clara, CA, USA) was used. The mobile phases were (A) CH_3CN and (B) formic acid/water pH 3.2, and flow rate 0.8 ml/min; temperature was set at $26\text{ }^{\circ}\text{C}$. The following gradient profile was used: 0-2 min, 5-15% A, 95-85% B; 2-5 min, 15% A, 85% B; 5-7 min 15-50% A, 85-50% B; 7-12 min, 50% A, 50% B; 12-15 min, 50-30% A, 50-70% B; 15-20 min, 30% A, 70% B; 20-25 min, 30-5% A, 70-95% B with equilibration time of 5 min. The chromatograms were acquired at 223 nm (for AG), 250 nm (for progesterone), 280 nm (for caffeine) and 350 nm (for piroxicam).

An HPLC-FLD method was used to quantify FITC ($\lambda_{\text{ex}}=492$ nm, $\lambda_{\text{em}}=518$ nm, green) of the fluorescent SLNs applied in the *in vivo* test. The column was a Lichrosorb C18 (4.6 mm x 100 mm i.d., 5 μm) maintained at 27 °C (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was (A) CH_3CN and (B) formic acid/water pH 3.2; flow rate 0.8 ml/min. The following gradient profile was used: 0-5 min, 10-40% A, 90-60% B; 5-10 min, 40-50% A, 60-50% B; 10-12 min 50-55% A, 50-45% B; 12-15 min, 55% A, 45% B; 15-18 min, 55-90% A, 45-10% B; 18-20 min, 10% A, 90% B with an equilibration time of 5 min.

To quantify NaF ($\lambda_{\text{ex}}=460$ nm, $\lambda_{\text{em}}=515$ nm, green), an HPLC-FLD method was used. The column was a Kinetex C18 (4.6 mm x 150 mm i.d., 5 μm) maintained at 27 °C (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was (A) CH_3CN and (B) formic acid/water pH 3.2; flow rate 0.8 ml/min and injection volume 10 μl . The following gradient profile was used: 0-3 min, 20% A, 80% B; 3-23 min, 20-80% A, 80-20% B; 23-25 min 80-100% A, 20-0% B; 25-27 min, 100-20% A, 0-80% B with an equilibration time of 5 min.

Standard solutions were freshly prepared by dilutions of stock solutions (0.5 mg/mL) in HPLC grade CH_3OH . An external standard method was applied to quantify each compound. Quantification of individual constituents was performed using a regression curve and the analyses were performed in triplicate.

All compounds showed a linear response: AG from 0.5 to 250 ng/mL, FITC from 6.40 to 520 ng/mL, and NaF from 0.5 to 460 ng/mL. All the curves had coefficients of linear correlation $R^2 \geq 0.999$.

A dilution of standard solutions was used to determine the limits of detection (LOD) ($S/N \geq 3$) and limits of quantification (LOQ) ($S/N \geq 10$). For AG, LOD was 2.6 ng and LOQ 5.3 ng.

Determination of yield%

The yield of the preparation process of SLNs was calculated as the weight of the product obtained after the freeze-drying compared to the weight of the components used in the reaction:

$$\text{Yield \%} = \frac{\text{Real weight (mg)}}{\text{Theoric weight (mg)}} \times 100$$

Stability studies

Stability of empty and AG-SLNs was studied over a one-month period in both nanoparticles as colloidal dispersion and lyophilized product. Aqueous dispersion was stored at 4 °C, while lyophilized SLNs were kept at room temperature (25 °C). At determined intervals, the samples were assayed for physical and chemical stability. Physical stability was checked by monitoring size and polydispersity by DLS; chemical stability was assessed by quantification of drug content by HPLC-DAD analysis.

SLNs were also incubated in a solution of human serum albumin (HSA) at body temperature under magnetic stirring to mimic *in vivo* conditions. Briefly, 200 µL of dispersion were exposed to 2 mL of albumin solution (40 mg/mL) for 2 h (Gualbert et al., 2003; Koziara et al., 2004).

A stability study of AG-SLNs was also performed in rat plasma. We collected whole blood into heparinized tubes. Cells are removed from plasma by centrifugation for 10 minutes at 1.000-2.000 x g using a refrigerated centrifuge. Following centrifugation, we immediately transferred the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette.

Stability of AG-SLNs and FITC-SLNs was evaluated by adding 250 µL of rat plasma to 50 µL of nanoparticles suspension. After 2 h of incubation, the resulted mixture was opportunely diluted for DLS analysis and particle size and PdI were measured.

In vitro release

In vitro release of AG and FITC from optimized formulations was carried out using dialysis membrane (Cellulose regenerated, cut-off 3.5 kD) in PBS/Ethanol 70:30 mixture, in absence and in presence of HSA at a concentration of 40 mg/mL to mimic *in vivo* conditions (Righeschi et al., 2016). Two millilitres of AG saturated solution (65 µg/mL in distilled water), AG solution (2.80 mg/mL in methanol), AG suspension (2.80 mg/mL in distilled water), AG-SLNs (30 mg/mL in distilled water, corresponding to 2.80 mg/mL of AG) or FITC-SLNs (15 mg/mL in distilled water) were inserted into pre-soaked dialysis tubes and placed in 200 mL of release medium. The medium was stirred at 37 °C. At pre-determined intervals, 1 mL of release medium was withdrawn and replaced with an equal volume of fresh release medium. The concentration of AG or FITC was defined by chromatographic analysis.

PAMPA studies

PAMPA studies were performed using the method reported in the literature (Di et al., 2003), optimized for our experiments. Caffeine, piroxicam and progesterone were used as standards and solubilized in ethanol at a concentration of 0.5 mg/mL and diluted five times in a mixture of PBS/Ethanol (70/30 v/v). A 96-well filter plate was used as the permeation donor compartment and a 96-well receiver plate was used as acceptor. A solution (2% w/v) of Porcine Polar Brain Lipid (PBL) in n-dodecane was prepared and the mixture was sonicated to ensure complete dissolution. PBL solution (5 µL) was added to each donor plate well (Di et al., 2003). Immediately after the application of the artificial membrane, 250 µL of the free drug or formulation were added to each donor compartment; the acceptor compartment was filled with PBS/Ethanol solution (250 µL). The drug-filled donor compartment was then placed in the acceptor plate.

After 18 h of incubation, samples were withdrawn from the donor and acceptor plates and standards or AG concentrations were assessed by HPLC-DAD analyses. In the case of the formulation, 150 μ L were withdrawn from both compartments, diluted with methanol and placed in the ultrasonic bath for 30 min, and finally ultracentrifuged for 1 h at $11330 \times g$ (4°C). The permeability of compounds was calculated using the following formula (Wohnsland & Faller, 2001):

$$P_e = -\ln[1 - C_A(t)/C_{\text{equilibrium}}] / A \times (1/V_D + 1/V_A) \times t$$

where P_e is permeability (cm/s), A = effective filter area ($f \times 0.3 \text{ cm}^2$), where f = apparent porosity of the filter, V_D = donor well volume (mL), V_A = receptor well volume (mL), t = incubation time (s), $C_A(t)$ = compound concentration in receptor well at time t , $C_D(t)$ = compound concentration in donor well at time t , and

$$C_{\text{equilibrium}} = [C_D(t) \times V_D + C_A(t) \times V_A] / (V_D + V_A)$$

Experiments were performed in triplicate and the mean of three samples was used for data analysis.

Blood-Brain Barrier hCMEC/D3 cell culture

Immortalized hCMEC/D3 cells were obtained from Millipore (Cat. # SCC066). This cell line is derived from human temporal lobe microvessels isolated from tissue excised during surgery for control of epilepsy. Cells were seeded in a concentration of 2.5×10^4 cells/cm² and grown at 37°C in an atmosphere of 5% CO₂ in 25 cm² rat tail collagen type I coated culture flasks. EndoGRO™- MV Complete Media Kit (Cat. # SCME004) supplemented with 1 ng/mL FGF-2 (Cat. #GF003) was changed every three days and cells were grown until they were 90% confluent. Cells were passaged at least twice before use. Confluent hCMEC/D3 cells were split by Accumax™ Cell Counting Solution in DPBS.

MTT assay

To assess cell viability after AG and AG-SLNs exposure, an MTT assay was performed. Cells were seeded in a 24-well plate (6×10^4 cells /cm²) pre-coated with Collagen Type I, Rat Tail (Cat. #08-115) and grown at 37°C in an atmosphere of 5% CO₂ in EndoGRO™ Basal Medium (EBM-2). When the cells were approximately 70-80% confluent, they were incubated with different concentrations of AG (5, 25 and 125 µM) and SLNs (0.3 and 0.06 mg/mL, obtained by redispersion in the EBM-2 of the lyophilized formulation) for 2 h. The SLNs formulation was previously filtered through 0.4 µm sterile filter units in order to maintain sterile conditions and minimize contaminations. The medium of each well was separated from the cells and stored for LDH assay, and cells were treated with 1 mg/mL of MTT for 1 h at 37 °C and 5% CO₂. Finally, DMSO was added to dissolve MTT formation and absorbance was measured at 550 and 690 nm. Cell viability was expressed as a percentage compared to the cells incubated only with EBM-2 (positive control). Triton X-100 was used in the MTT assay as the negative control since detergent action disrupts the cells.

LDH assay

The LDH assay was performed to determine cytotoxicity after AG and SLNs exposure. The medium resulting from incubation of AG and SLNs with cells was centrifuged (250 g, 10 min at RT) and the supernatant separated from the deposited cells in each well. This centrifugation process allowed us to remove any wastes and cellular debris as well as AG and SLNs. The release of LDH into culture supernatants was detected by adding catalyst and dye solutions of a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, USA). The absorbance values were read at 490 nm and 690 nm. Cytotoxicity was expressed as a percentage compared to the maximum LDH release in the presence of Triton X-100 (positive control). EBM-2 was used as the negative control since no cytotoxicity was detected in such conditions.

hCMEC/D3 cell culture for transwell permeability studies

For all transcytosis assays, high-density pore (2×10^6 pores/cm²) transparent PET membrane filter inserts (0.4 μ m, 23,1 mm diameter, Falcon, Corning BV, Netherlands) were used in 6-well cell culture plates (Falcon, Corning, Amsterdam, The Netherlands). Before the cell barrier coating, the transparent PET membrane filter inserts were coated with rat tail collagen type I at a concentration of 0.1 mg/mL and incubated at 37 °C for 1 h. Inserts were subsequently washed with PBS and incubated for 1 hour after which time PBS was removed and replaced with the assay medium. The inserts were calibrated with the assay medium at 37 °C for at least 1 hour. Optimum media volumes were calculated to be 1 mL and 1.2 mL for apical and basolateral chambers respectively. After the transwell inserts were calibrated with assay medium for 1 h, the medium was removed and hCMEC/D3 cells seeded onto the apical side of the inserts at a density of 6×10^4 cells /cm² in 1 mL assay media. 1.2 mL of fresh medium was added to the basolateral chamber. The assay medium was changed every three days following transwell apical insert seeding with hCMEC/D3. Cells were grown to confluence for seven days. hCMEC/D3 monolayers were used as a permeability assay for AG and AG-loaded SLNs. Fluorescein sodium salt (NaF) at a concentration of 10 μ g/mL was used as integrity control marker with a known permeability coefficient (P_{app}) for this cell line (Eigenmann et al., 2013). The integrity of monolayer cells was also confirmed by observation of cultures under phase-contrast microscopy or bright-field optics using of transparent membranes. The image was viewed using an inverted microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) with a low-power objective (20X). Images were digitized using a video image obtained with a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA).

For permeability studies, AG (5, 25 and 125 μM) and AG-SLNs (0.3 mg/mL, corresponding to AG 80 μM) obtained by redispersion in the EBM-2 of the lyophilized formulation were tested and incubated in the apical donor compartment for 1 h. At the end of incubation, the amount of NaF and AG were quantified both in apical and basolateral compartments by HPLC-FLD or HPLC-DAD method. In the case of the formulation, EBM-2 was diluted with methanol and placed in the ultrasonic bath for 30 min, then ultracentrifuged for 1 h at 11330 $\times g$ (4 $^{\circ}\text{C}$). The apparent permeability coefficients (P_{app}) of free AG and AG encapsulated in SLNs was calculated according to the equation (Youdim et al., 2003):

$$P_{\text{app}} (\text{cm/s}) = V_{\text{D}} / (A M_{\text{D}}) \times (\Delta M_{\text{R}} / \Delta t)$$

where V_{D} = apical (donor) volume (cm^3), M_{D} = apical (donor) amount (mol), $\Delta M_{\text{R}}/\Delta t$ = change in amount (mol) of compound in receiver compartment over time.

Recovery for AG and NaF was calculated according to the equation (Eigenmann et al., 2013):

$$\text{Recovery (\%)} = C_{\text{Df}} V_{\text{D}} + C_{\text{Rf}} V_{\text{R}} / (C_{\text{D0}} V_{\text{D}}) \times 100$$

where C_{Df} and C_{Rf} are the final concentrations of the compound in the donor and receiver compartments, C_{D0} is the initial concentration in the donor compartment and V_{D} and V_{R} are the volumes in the donor and receiver compartments, respectively. All experiments were performed at least in triplicate.

In vivo experiments

One-month-old, 100-150 g Wistar rats (Harlan, Milan, Italy) (n = 3-4/group) were used, divided into the following groups:

group 1): sacrificed after 3 h from injection of 200 μ L of nanoparticles

group 2): sacrificed after 24 h from injection of 200 μ L of nanoparticles

group 3): sacrificed after 72 h from injection of 200 μ L of nanoparticles

group 4): controls (200 μ L of saline)

group 5): sacrificed after 3 h from injection of FITC solution (200 μ L of 0.1 mg/mL of FITC dissolved in saline)

Animals were housed in macrolon cages with ad libitum food and water and maintained on a 12-h light/dark cycle at 23 °C. All experiments were carried out according to the EC Directive 86/609/EEC for animal experiments and national guidelines for animal care (Permit Number: 152/2014-B). All efforts were made to minimize the number of animals used and their suffering.

Intravenous administration of fluorescent SLNs

To evaluate the ability of SLNs to cross the BBB, rats were acutely dosed with SLNs, FITC solution or vehicle (0.9% NaCl) by intravenous injection via the tail vein. At the time of sacrifice, rats were perfused transcardially with 500 mL of saline solution (0.9 %) to remove blood components and then with 4 % ice-cold paraformaldehyde in 0.1 M PBS, pH 7.2, under deep anaesthesia (chloral hydrate, 400 mg/kg, i.p.). After sacrifice, brains were quickly extracted and fixed in 4% paraformaldehyde for 24 h. Subsequently, brains were rinsed with PBS for 24 h, dehydrated using an automated machine, and embedded in paraffin. Coronal sections (5.0 μ m) were cut using a microtome and mounted on slides.

Immunohistochemistry

Immunohistochemistry was performed on 5.0 µm coronal paraffin-embedded sections, as described in the literature (Grossi et al., 2017). The sections were incubated overnight at 4 °C with the primary antibody (Ab) anti-CD34 (1:50) and anti-IBA1 (1: 200) diluted in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100 and 5.0 mg/mL BSA. On day 2, sections were incubated for 2 h in the dark with the appropriate fluorescent secondary Ab (Alexa Fluor 594 conjugated monoclonal anti-mouse and polyclonal anti-rabbit Ab; Invitrogen, New York, USA) diluted 1:400. At the end of incubation, sections were washed in PBS-Triton and subsequently covered with a coverslip using a specific aqueous upright for viewing preparation by the microscope in fluorescence (Vectashield mounting medium plus nuclear marker DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), VectorLaboratories, Burlingame, CA, USA). Sections were then analyzed using an epi-fluorescence microscope connected to a digital camera (Olympus BX63, Milan, Italy) and the most significant images were acquired using the CellSensDimension Software (Olympus, Milan, Italy).

Statistical analysis

Experiments were repeated three times and results expressed as a mean ± standard deviation. Statistical significance was calculated by Student's t-test with statistical significance level set at $P < 0.05$.

RESULTS AND DISCUSSION

Preparation and characterization of SLNs

Nanoparticles have emerged as versatile, biodegradable, biocompatible, non-antigenic nano-carriers for the specific and targeted delivery of drugs to organs and tissues, which is particularly relevant in cancer therapy where most of the biological processes occur at the nanometer level (Yang et al., 2013; Metwally et al., 2016). Many of these studies are very promising and they have resulted in nanoformulations with sustained release and improved bioavailability at much lower doses than conventional preparations, and in many cases presenting a better safety profile.

In this context, we designed SLNs using Compritol and brij 78 to deliver AG to the brain and to overcome its biopharmaceutical limitations. The components under study were selected for their biocompatibility, biodegradability, and versatility (J. Q. Zhang et al., 2007; Sanchez-Lopez et al., 2017).

Compritol is a mixture of glycerol with behenic acid esters, while brij 78 is a non-ionic and non-toxic surfactant, conferring “stealth” characteristic to the nanoparticles, with the possibility to realize a constant and prolonged release (J. Q. Zhang et al., 2007). Different lipid/surfactant ratios were tested (Table 1).

In the presence of a greater quantity of lipid, nanoparticles were larger (300 nm), highly polydispersed and not suitable for parenteral administration. Nanoparticles became more homogeneous by increasing surfactant amount. SLNs obtained with the gravimetric ratio 1:3 showed the best technological parameters and they resulted suitable for administration by the intravenous route (Table 1). Their high ζ -potential confirmed the stability of the system.

Compritol : Brij 78 (w/w)	Size (nm)	Pd	ζ -potential (mV)
2.5:1	321 $\pm$ 7	0.28 $\pm$ 0.01	-31.2 $\pm$ 0.4
2:1	408 $\pm$ 5	0.44 $\pm$ 0.09	-30.2 $\pm$ 0.8
2:1.5	384 $\pm$ 28	0.49 $\pm$ 0.06	-32.2 $\pm$ 1.0
1:1.5	293 $\pm$ 7	0.23 $\pm$ 0.02	-32.2 $\pm$ 0.7
1:2	283 $\pm$ 4	0.21 $\pm$ 0.01	-36.6 $\pm$ 0.6
1:3	266 $\pm$ 4	0.18 $\pm$ 0.01	-36.4 $\pm$ 0.5

Table 1. Physical characterization of empty SLNs obtained with different Compritol : Brij 78 w/w ratios.
Data displayed as mean $\pm$ SD; n=3.

Sample	Size (nm)	PdI	ζ -potential (mV)	EE%	LC%
SLN	266 $\pm$ 4	0.18 $\pm$ 0.03	-36.4 $\pm$ 6.3	-	-
FITC-SLN	278 $\pm$ 10	0.18 $\pm$ 0.01	-36.1 $\pm$ 0.9	78.7 $\pm$ 0.7	3.7 $\pm$ 0.1
AG-SLN 2.5%	278 $\pm$ 5	0.19 $\pm$ 0.03	-29.6 $\pm$ 0.3	81.2 $\pm$ 1.0	1.9 $\pm$ 0.2
AG-SLN 5%	264 $\pm$ 2	0.13 $\pm$ 0.04	-30.4 $\pm$ 0.5	86.0 $\pm$ 1.8	4.2 $\pm$ 0.1
AG-SLN 10%	262 $\pm$ 7	0.18 $\pm$ 0.03	-36.0 $\pm$ 4.1	92.4 $\pm$ 1.8	8.4 $\pm$ 0.2

Table 2. Physical and chemical characterization of empty, AG and FITC-loaded SLNs.
Data displayed as mean $\pm$ SD; n=3.

Different percentages (2.5%, 5% and 10%) of the drug were loaded to obtain AG-SLNs. Nanoparticles with AG 10% have similar characteristics to the empty carrier (Table 2). For this level, EE% and LC% were of $92.4\% \pm 1.8\%$ and $8.4\% \pm 0.2\%$ respectively. TEM images confirmed DLS results and they reveal that all nanoparticles were nearly spherical and uniform in shape (Figure 1).

In view of an *in vivo* administration, fluorescent nanoparticles (5% of FITC, Makarova et al., 1999) were prepared, using the same method described for AG-SLNs. Fluorescent SLNs maintained dimensions below 300 nm with a proper size distribution suitable for parenteral administration (Table 2), with EE% and LC% resulting as $78.7\% \pm 0.7\%$ and $3.7\% \pm 0.1\%$, respectively.

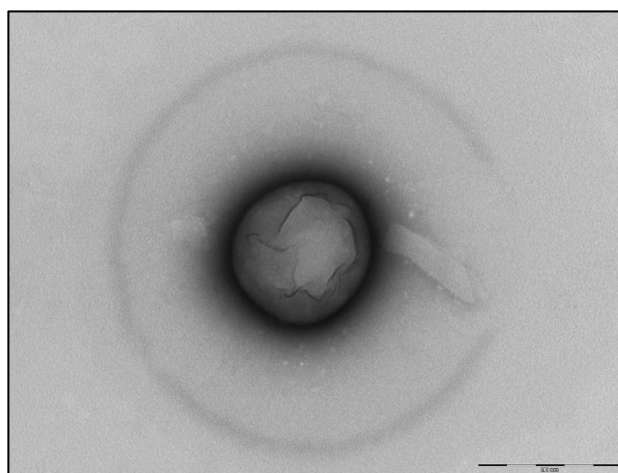


Figure 1. TEM image of AG-loaded SLNs.

Scale 200 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Stability studies

The conservation of SLNs as lyophilized product offers advantages concerning the stability and formulation versatility. Toward this aim, AG-SLNs were lyophilized overnight, producing a voluminous and spongy solid which was easily dispersible in water. The yield percentage of the whole preparative process, including the lyophilization, was $99\% \pm 1$, EE% and LC% resulted $92\% \pm 0.1\%$ and $8.3\% \pm 0.1\%$ respectively.

As lyophilized powder or an aqueous dispersion, AG-SLNs were maintained at a temperature of 4°C and 25°C , respectively, for one month and analyzed at regular intervals. No significant changes were observed in terms of size, Pdl and ζ -potential values for both the products (Figure 2).

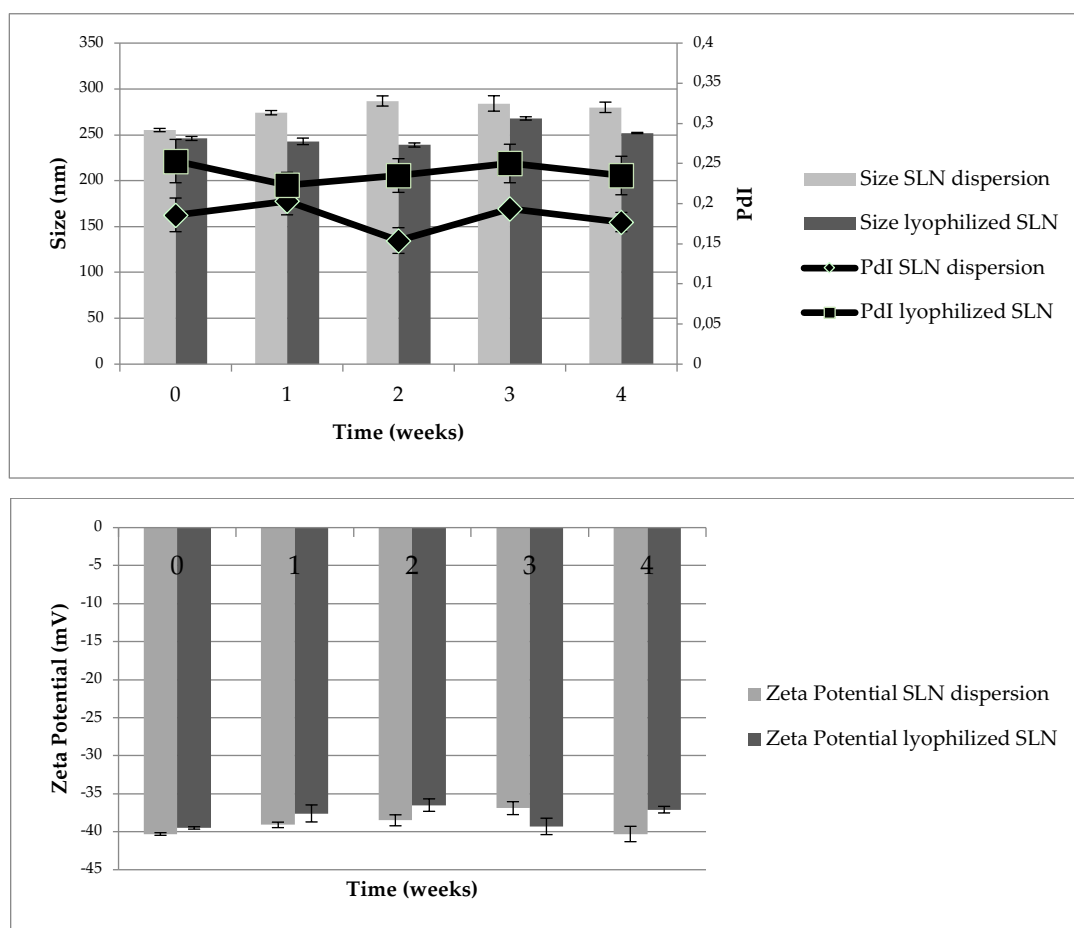


Figure 2. Particle size, Pdl and ζ -potential of AG-SLNs as dispersion or lyophilized product after one-month storage at 4°C and 25°C .

Data displayed as mean $\pm$ SD; $n=3$.

In addition, the EE% remained close to 92-95% (Figure 3). The presence of the surfactant gives a high ζ -potential value that prevents precipitation and aggregation of nanoparticles.

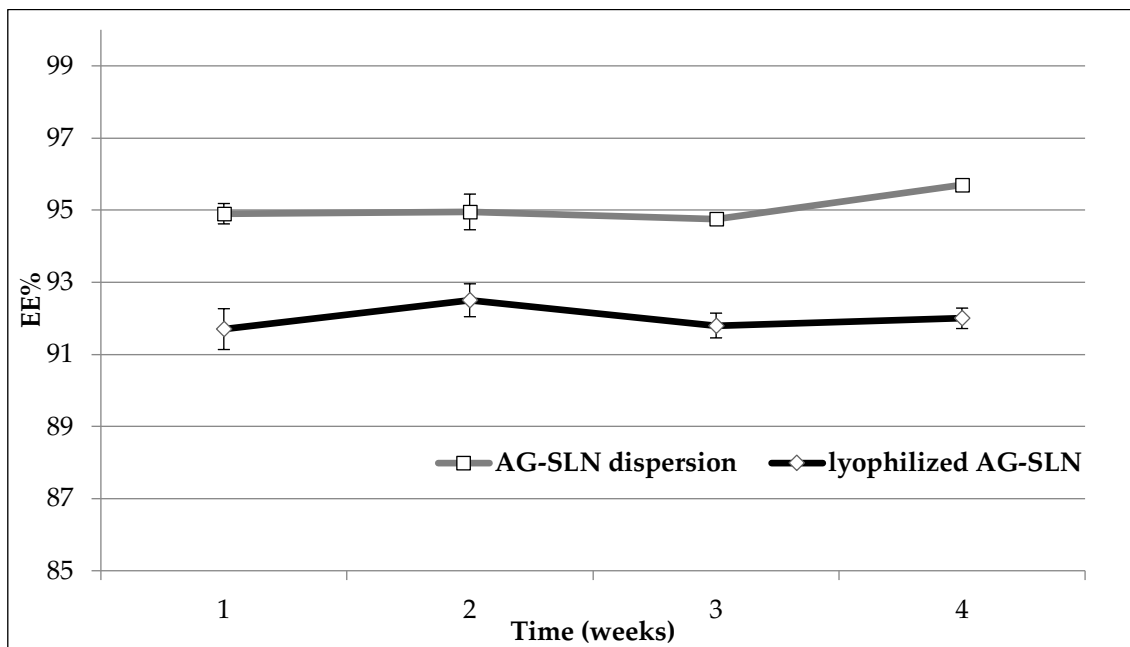


Figure 3. Chemical stability (EE%) of AG-SLNs during storage at 4°C and 25°C.
Data displayed as mean $\pm$ SD; n=3.

The stability of SLNs in the presence of HSA at physiological concentration was also evaluated. DLS analysis confirmed that sizes were not affected, thus revealing a coexistence of free serum proteins and optimized nanocarrier without any protein corona effect (Isacchi et al., 2012; Table 3). This result can be attributed to the protective action of the hydrophilic PEG chain present in the chemical structure of brij 78.

In the case of stability in rat plasma, no aggregation phenomena occurred after 2 h of incubation. DLS analysis revealed that particle size remained around 260 nm (262 ± 5) for AG-SLNs and 270 nm (273 ± 5) for FITC-SLNs and no significant changes in PDI values were observed (0.20 ± 0.01 and 0.19 ± 0.02 , respectively), confirming the good results obtained in the presence of HSA solution and stealth properties of developed SLNs.

Sample	Time	Size (nm)	PdI
AG-SLNs	initial	260 ± 5	0.18 ± 0.03
	30'	272 ± 3	0.21 ± 0.03
	1 h	270 ± 5	0.23 ± 0.05
	2 h	278 ± 3	0.21 ± 0.01
FITC-SLNs	initial	278 ± 10	0.18 ± 0.02
	30'	256 ± 5	0.19 ± 0.01
	1 h	248 ± 8	0.21 ± 0.01
	2 h	249 ± 5	0.21 ± 0.01

Table 3. Particle size and polydispersity index (PdI) of AG-SLNs and FITC-SLNs in the presence of human serum albumin.

Data displayed as mean $\pm$ SD; n = 3.

DSC studies

DSC analyses were performed to assess drug-lipid interactions (Muller et al., 2000). The following samples were analyzed: pure AG, Compritol, brij 78, a physical mixture of empty SLNs and AG, and AG-SLNs. Figure 4 depicts the DSC thermograms obtained. The observed melting peak at 72°C might be due to a mixture of metastable polymorphic β and β' forms of Compritol. DSC analysis of AG indicates the melting point at 230°C and at 46°C for brij 78. In the thermogram of AG-SLNs, an AG melting peak was not recorded due to its amorphisation or nearly complete molecular dispersion into the lipid matrix. As previously reported, the absence of a melting peak for a crystalline drug is due to its solubilization and/or amorphisation in the nanoparticle formulation (Righeschi et al., 2016). On the contrary, the melting peak of Compritol was detectable both in empty and in AG-SLNs, even though it appeared clearly reduced in intensity and slightly shifted, indicating a loss of crystallinity with respect to the pure lipid. This result is due both to the presence of other components in the formulation and to the preparation method of nanoparticles (Bunjes & Unruh, 2007; Righeschi et al., 2016).

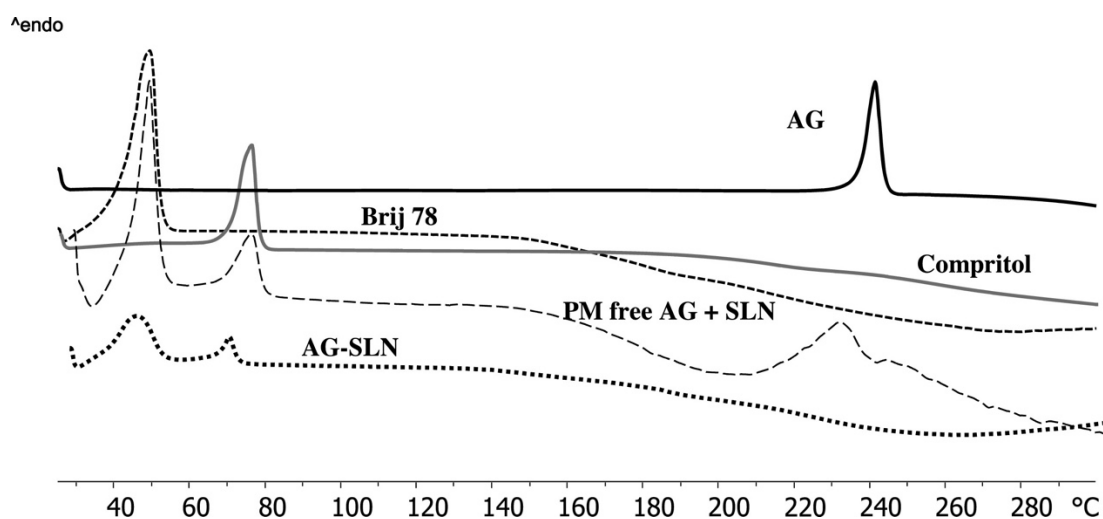


Figure 4. DSC curves of pure components lyophilized AG-SLNs and mixture of empty SLNs and AG.

To exclude that AG is present as a crystalline compound in the SLNs, we also analyzed the mixture of empty SLNs and AG. In this case, the AG melting peak is visible, demonstrating that the drug is present in an amorphous state when loaded into SLNs.

In vitro release

The dialysis bag method was used to evaluate the AG and FITC *in vitro* release from optimized formulations (Kakkar et al., 2011; Righeschi et al., 2016). The release profiles (Figure 5) of AG saturated solution in water, AG solution in methanol, AG suspension in water, AG-SLNs in water and in the presence of HSA are reported.

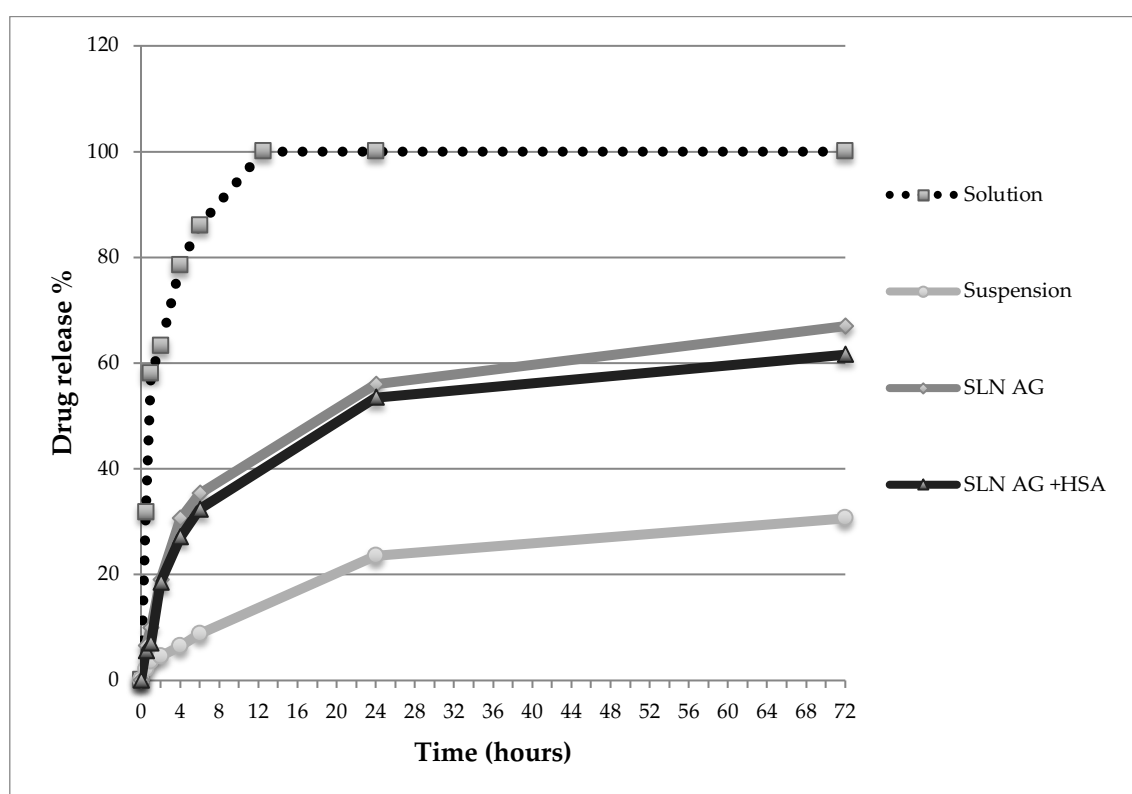


Figure 5. *In vitro* release profiles of AG solution, AG saturated solution, AG suspension, AG-SLNs and AG-SLNs in the presence of HSA. Data displayed as mean $\pm$ SD; n = 3.

The release rate of AG from SLNs was slower than that from the solutions and faster than that from the suspension, as also reported in the literature (Yang et al., 2013). In the case of the saturated aqueous solution, about 50% of AG was released after 4 h; the percentage increased to 75.3% at 24 h and reached 85.6% at 72h. 86.7% of AG was released from methanol solution at 4 h, whereas the percentage decreased to 35.3% at 6 h for AG-SLNs, and the release was prolonged for approximately 72 h until a percentage of 67.0%. The release of AG from suspension was slower compared with AG-SLNs, only 15.6% of AG was released within 72 h.

AG-SLNs showed a rapid release (burst release) in the first six hours. This result is due to the fast release of the adsorbed and/or precipitated molecules from the drug-enriched shell around the particles. In the remaining time, a slower release over 72 h (referred to as sustained release) was observed. During this time AG dispersed within the core was released slowly from the lipid matrix. A slow release was expected due to the solid nature of the nanoparticles (Dan, 2014). In this context, the use of SLNs increases the solubility of AG and guarantees a prolonged release. Finally, given the low aqueous solubility of AG (0.07 mg/mL), its encapsulation into SLNs allows the administration of the molecule at a higher concentration (30 mg/mL contained 2.80 mg/mL of AG), an important aspect for parenteral administration and for a possible reduction of the administered dosage with the aim to decrease the toxicity of AG at high dosages, as reported in the literature (Yen et al., 2013).

In the presence of HSA, the AG release from SLNs remains functional for a sustained release of the molecule and is identical to release without HSA.

The release of FITC from fluorescent SLNs was evaluated to determine the stability of the probe within nanoparticles during the *in vivo* experiments. The test confirmed that FITC is not released until 24 hours (Figure 6).

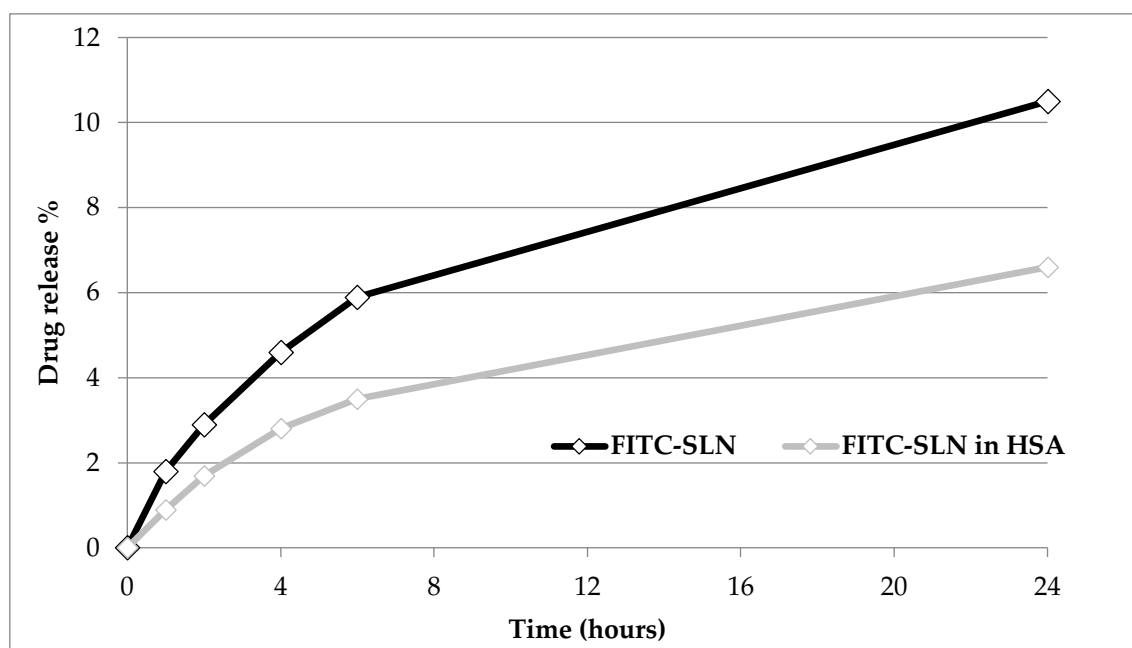


Figure 6. *In vitro* release profiles of FITC-SLNs and FITC-SLNs in the presence of human serum albumin (HSA). Data displayed as mean $\pm$ SD; n=3.

PAMPA studies

PAMPA assay is a particularly helpful complement to the cellular permeability model for its speed, low cost and versatility, and readily provides information about passive-transport permeability. Different solvents (DMSO, MeOH, EtOH and PBS) were considered to optimize the test for standard compounds. Ethanol resulted the best one, as also reported by Salado et al., 2014. After the incubation time (18 h), solutions were taken from acceptor and donor compartments and analyzed to estimate the permeability values (P_e). The values obtained for standards reflected the classification reported in the literature for PAMPA-BBB test (Makarova et al., 1999): caffeine and piroxicam have low permeability and progesterone resulted as a compound with high BBB permeability.

In PAMPA-BBB assay, AG resulted as a molecule with low permeability, with a P_e value of $0.487 \pm 0.155 \times 10^{-6}$ cm/s and therefore a perfect candidate to be encapsulated into SLNs. P_e of AG loaded into SLNs was found to be increased ($3.25 \pm 0.09 \times 10^{-6}$ cm/s). The value confirmed that nanoparticles also increased the permeability of AG compared to AG alone in aqueous solution.

MTT and LDH assays

MTT and LDH assays were performed in hCMEC/D3 cell line to evaluate the effect of AG and AG-SLNs in cell viability and cytotoxicity, and permeability studies were also conducted in transwell devices using the same cell line. The *in vitro* cytotoxicity of the developed SLNs and AG was assessed by cell viability determination and membrane integrity evaluation using the hCMEC/D3 cell line in MTT and LDH assays, respectively (Figure 7).

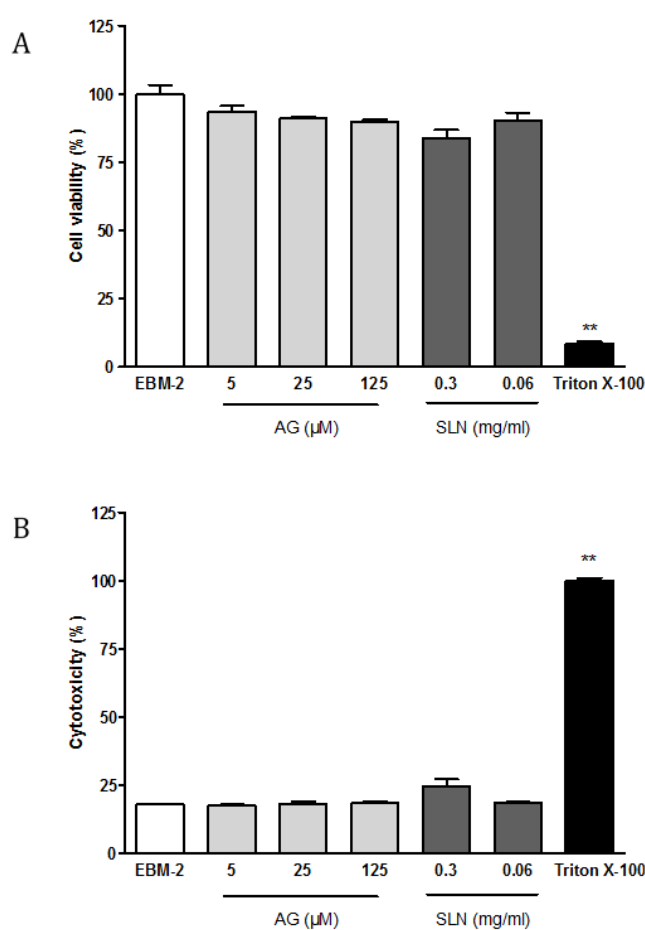


Figure 7. hCMEC/D3 cell viability evaluated by MTT assay (A) and cytotoxicity by LDH assay (B) when exposed for different time periods (2, 6 and 24 h) to AG (25 and 125 μ M) or AG-SLN (0.06 and 0.3 mg/mL).

Data are expressed as percentage of control (EBM-2 medium) and Triton-X (TTX) which represent, respectively, the maximum cell viability and cell cytotoxicity. Values represent the mean $\pm$ SEM of at least three experiments performed in triplicate.

* $P < 0.05$ and ** $P < 0.01$ vs. EBM-2 alone.

When cells were exposed to different concentrations of AG (25 and 125 μ M) and AG-SLNs (0.06 and 0.3 mg/ml) for 2 and 4 h, no significant changes were observed in MTT metabolization or LDH release when compared to cells exposed to the EBM-2 medium alone, indicating that AG and AG-SLNs affected neither the metabolic activity of the cells nor the membrane integrity at these time points. On the other hand, when the cells were incubated for 24 h with AG at a dose of 125 μ M and AG-SLNs (0.06 and 0.3 mg/ml) we observed cellular death. These data are in accordance with Yen and colleagues who observed AG induced cellular toxicity in a dose-dependent manner in CEC cell lines (Yen et al., 2013). The same authors evidenced *in vivo* that AG at a rather low dose (0.1 mg/kg) protects against brain injury, but worsens it at a higher dose (5 mg/kg).

BBB permeability studies

hCMEC/D3 brain microvascular endothelial cell line is an easily grown model of human BBB that is amenable to cellular and molecular studies on pathological and drug transport mechanisms with relevance to the CNS (Poller et al., 2008; Vu et al., 2009). hCMEC/D3 cells retain the expression of most transporters and receptors expressed *in vivo* at the human BBB.

hCMEC/D3 permeability coefficients (P_{app}) correlate well with *in vivo* permeability data therefore permeability studies were performed to predict the permeability of free AG and AG-SLNs across the BBB. NaF was used as negative control and its P_{app} was determined during all transport experiments in the absence and in the presence of AG or AG-SLNs to monitor the integrity of the cell layer. The integrity was also assessed by observation of cultures under phase-contrast microscopy or under bright-field optics using transparent membranes.

The P_{app} of NaF after 1 h was $11.1 \pm 1.8 \times 10^{-6}$ cm/s, in agreement with the literature value ($12.5 \pm 0.3 \times 10^{-6}$ cm/s; Eigenmann et al. 2013; Righeschi et al., 2016). This data confirms the confluence of the monolayer and assesses the tight junction integrity of the cell layer because this fluorescent molecule has low BBB penetration and it was used as barrier integrity marker for *in vivo* models. Permeability results (P_{app} values) after 1 h of experimentation are reported in Table 4.

Sample	P_{app} ($\times 10^{-6}$ cm/s)		
NaF	11.1 ± 1.8		
AG 5 μ M	N.D.	NaF	11.3 ± 0.7
AG 25 μ M	10.2 ± 1.7	NaF	13.2 ± 1.9
AG 125 μ M	14.5 ± 2.9	NaF	12.4 ± 1.8
AG-SLNs	26.8 ± 2.2	NaF	12.7 ± 0.7

Table 4. Apparent permeability coefficients (P_{app}) for AG at different concentrations, for AG-loaded SLNs and for NaF as barrier integrity control.
Data displayed as mean $\pm$ SD; n=3.

The first experiments were done with free AG at 5, 25 and 125 μ M, with co-incubation with NaF at 10 μ g/mL as negative control. The P_{app} of AG 5 μ M for the apical to basolateral transport could not be calculated because AG was below the limit of detection. At higher concentrations, P_{app} of AG was $10.2 \pm 1.7 \times 10^{-6}$ cm/s and $14.5 \pm 1.7 \times 10^{-6}$ cm/s for 25 and 125 μ M concentrations, respectively, proving that the molecule did not significantly permeate the hCMEC/D3 cell monolayer because its P_{app} value was similar to that of NaF.

After one hour of transport across the hCMEC/D3 cell monolayer (Guccione et al., 2017), the P_{app} value recorded with SLNs loaded with 80 μ M of AG significantly increased the permeability of AG, reaching a value of $26.8 \pm 4.2 \times 10^{-6}$ cm/s, about three-fold higher than for free AG. This data correlated well with the PAMPA results. The P_{app} of negative control remained unchanged and barrier integrity was preserved during the test.

Furthermore, during the experiment, the hCMEC/D3 monolayer remained intact with no relevant morphological changes, as also demonstrated by observation of cultures under phase-contrast microscopy or under bright-field optics before and after each permeability test (Figure 8). The recovery of AG and NaF was above 80% in all experiments, suggesting an acceptable *in vitro* prediction of P_{app} values. As reported in the literature (Poller et al., 2008; Neves et al., 2017), the main mechanism involved in the SLNs uptake by hCMEC/D3 cells was found to be clathrin-mediated endocytosis. Nanoparticles may partially follow lysosomal and endosomal trafficking inside cells. In fact, the lysosomal pathway is responsible for particle degradation and release of drug content inside cells, while the endosomal trafficking may be involved in the transport of intact drug-loaded NPs from one side to the other of the cell barrier.

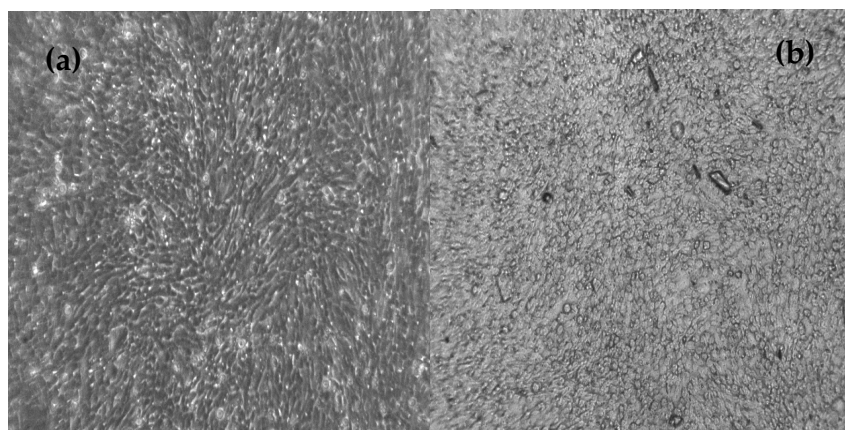


Figure 8. Microscopy images of hCMEC/D3 cell monolayer after (a) and before (b) the permeation *in vitro* test with AG-SLNs.

The stability of AG-SLNs during the experiment was assessed by TEM analyses of the medium of apical and basolateral compartments at the end of the assay. The images (Figure 9) confirmed the integrity of nanoparticles after permeation through the cell layer and the endocytosis as the main transport mechanism.

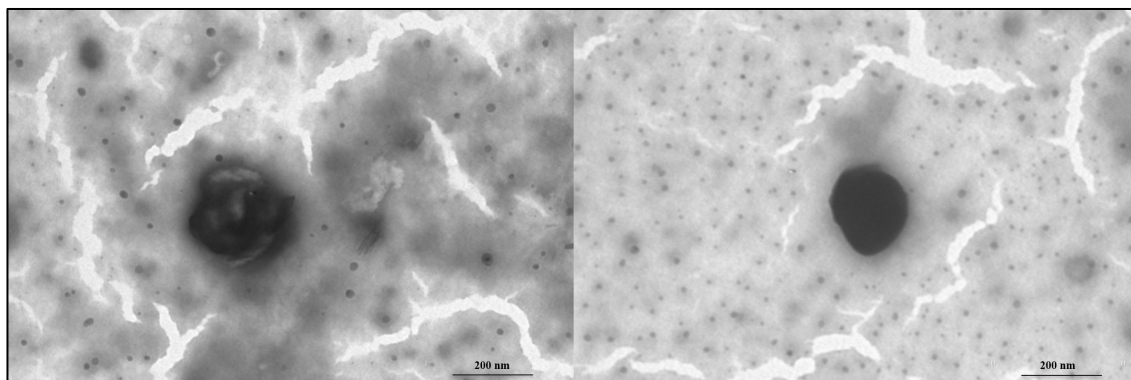


Figure 9. TEM images of AG-SLNs into apical (**left**) and basolateral (**right**) compartment after 1 h of the *in vitro* permeation test. Scale 200 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

In vivo experiments

Observation of fluorescent SLNs localization after administration into the caudal vein of rats was carried out by analyzing preparations under a fluorescence microscope. Initially, the presence of SLNs was evaluated in groups of rats sacrificed at 3, 24 and 72 h after injection (Figure 10 A). Based on the histochemical analysis, with DAPI (blue), a high presence of SLNs (green) was observed in the brain of animals sacrificed 24 h after injection (Figure 11). In animals sacrificed 3 and 72 h after injection, a smaller number of SLNs was identified probably due to the fact that in the few hours from administration, the "long circulating" nanocarriers are located predominantly into the bloodstream and after three days they are mostly degraded. In the control group (saline) no green fluorescence was observed, thus confirming the presence of SLNs in the other groups and excluding a possible autofluorescence of the tissue. Subsequently, in the group of rats sacrificed at 24 h and characterized by an abundant presence of nanoparticles, the localization of SLNs was further evaluated.

By immunohistochemical analysis with CD34 antibody, an endothelial cell marker, nanoparticles were found both at vessel level (white arrows) and in brain parenchyma (yellow arrows) (Figure 10 B).

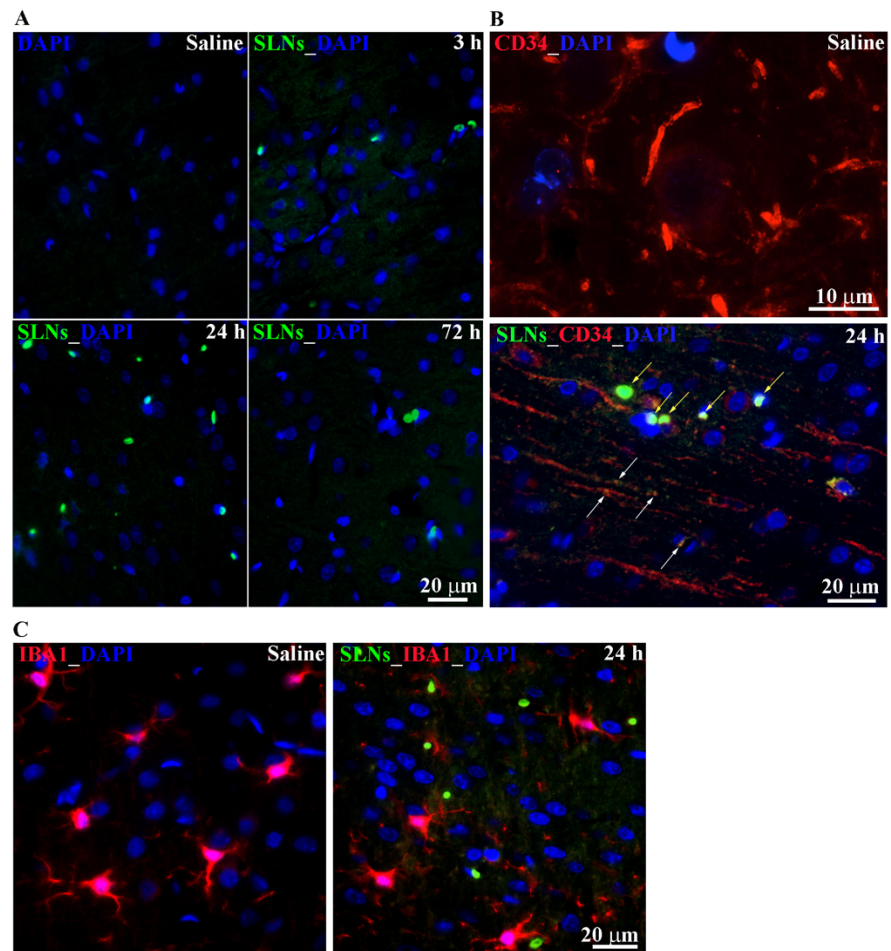


Figure 10. A) Intravenous administration of FITC-SLNs: representative photomicrographs showing the presence of SLNs (green) in brain parenchyma of rats 3, 24 and 72 h after injection.

Note the high presence of fluorescent SLNs at 24 h after injection.

Scale bar 20 μm applied to all images.

B) Immunohistochemical detection of SLNs (green) with the endothelial marker CD34 (red) in the brain of rats 24 h after injection. SLNs are detected both within blood vessels (white arrows) and brain parenchyma (yellow arrows).

A-B) No SLNs are detected in the brain of rats injected with saline.

Scale bar 20 μm applied to both images.

C) Immunohistochemical detection of SLNs (green) with the microglia antibody IBA1 (red) in the brain of rats 24 h after injection. SLNs are not detected within microglial cells and no microglia activation is detected compared to saline injected rats.

Scale bar 20 μm applied to both images. A-C) DAPI is in blue.

In the saline-injected rats (Fig. 10 B) no fluorescence was detected in either the vessels or brain tissue, further excluding the presence of nonspecific tissue autofluorescence in the SLNs-administered rats.

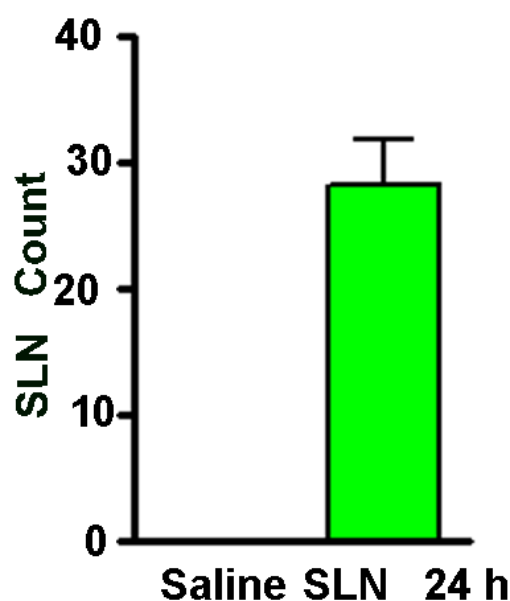


Figure 11. Quantitative analysis of FITC-SLNs in the somatosensory cortex of rats 24 hours after intravenous injection by the tail vein of 200 μ L of fluorescent SLN or saline.

Data are reported as mean $\pm$ S.E.M.

SLNs were counted under a 20 $\times$ objective lens, using an Olympus BX63 microscope, in the somatosensory cortex of rats 24 h after intravenous injection by the tail vein of 200 μ L of SLNs.

Five sections per animal, spaced 50-100 μ m from one another, were analyzed.

The total number of SLNs was averaged and reported as mean $\pm$ standard error of the mean (S.E.M). Saline (200 μ L) injected rats are reported as controls (n=3/group).

Data are analyzed using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

In the FITC solution injected rats some fluorescence was detected within few brain vessels (arrow) but not in the brain parenchyma (Figure 12). Then, by means of IBA1 antibody that recognizes microglial cells, we evaluated whether the administration of SLNs leads to any microglia activation.

As shown in Figure 10 C, the morphology of microglial cells (red) in different brain areas of rats, sacrificed 24 h after intravenous injection of SLNs (green), is typical of animals at rest and not different from that of saline-injected rats. This lack of microglia activation likely indicates that the brain macrophages do not recognize SLNs as foreign bodies.

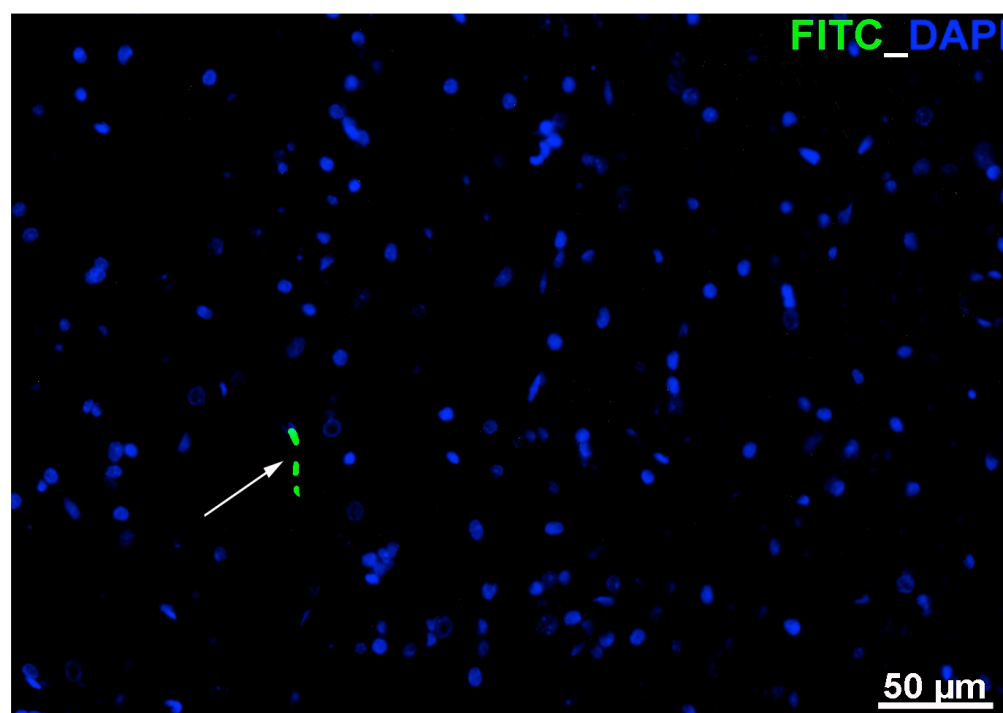


Figure 12. A representative photomicrograph showing FITC (green) within a blood vessel (arrow) in the brain of FITC solution injected rats, DAPI in blue.

These findings in agreement with data reported in the literature about the transport mechanism of SLNs across the BBB (Poller et al., 2008). The relative strategy employed by SLNs involves endocytosis influenced by developing concentration gradient followed by membrane fluidity and transcytosis of particles through the endothelium.

Kaur et al., 2008 reported that SLNs may likely permeate the BBB. Once the nanoparticle enters the endothelium it has to counteract the microvascular environment, which consists of astrocytes, pericytes, neurons, and extracellular matrix in order to cross the BBB. Our formulation possesses a highly lipophilic characteristic and charge, which helps the nanoparticles overcome astrocyte and pericyte attack, thereby facilitating the permeation of drug across the neurovascular junction.

Altogether, these preliminary data indicate that the formulation containing “stealth” SLNs can pass the BBB and reach the brain tissues that do not recognize them as a foreign agent. In fact, the surface coating with brij 78 reduces opsonization, phagocytosis, and clearance by the liver and reticuloendothelial system; consequently, the retention time of SLNs in plasma was increased. Furthermore, the improved blood residence time in brain capillaries, with absorption to the capillary walls, creates a higher concentration gradient that enhances penetration across the endothelial cell layer. In addition, SLNs could cross the BBB intact by transcytosis (J. X. Wang et al., 2002; Kaur et al., 2008; Barbu et al., 2009; Loureiro et al., 2017)

CONCLUSIONS

Novel nanoformulations based on drug delivery systems offer significant promise in overcoming the limitations of AG. In the present study, we have established that stealth-SLNs were successful in enhancing AG permeation using *in vitro* BBB models, PAMPA, and hCMEC/D3 cells. Furthermore, the ability of nanoparticles to cross the BBB and reach brain tissues was confirmed by *in vivo* test in healthy rats. SLNs have physical characteristics for systemic administration in terms of particle size, polydispersity, encapsulation efficacy, and ζ -potential. *In vitro* drug release studies have revealed that SLNs release AG in a sustained and controlled manner. SLNs show excellent chemical and physical stability as both suspension and lyophilized product. *In vitro* transport studies performed with PAMPA and hCMEC/D3 cells revealed that SLNs were successful in enhancing the permeation of AG while *in vivo* studies confirmed that nanoparticles could cross the BBB and reach the brain tissues. This suggests that the developed nanocarriers represent new potential brain delivery systems to increase the efficacy of AG in the field of neurodegenerative diseases.

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hCMEC/D3 cells studies were performed in collaboration with Prof. Domenico E. Pellegrini-Giampietro and Dr. Elisa Landucci (Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).

In vivo studies were performed thanks to the collaboration with Prof. Fiorella Casamenti, Dr. Daniela Pantano and Dr. Pamela Nardiello (Department of Neuroscience, Psychology, Drug Research and Child Health Division of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).



Full Length Article

Solid lipid nanoparticles for delivery of andrographolide across the blood-brain barrier: *in vitro* and *in vivo* evaluation



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Graverini, G.*, **Piazzini, V.***, Landucci, E., Pantano, D., Nardiello, P., Casamenti, F., ... & Bergonzi, M. C. (2018).

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***These authors contribute equally to this work**

STEALTH AND CATIONIC NANOLIPOSOMES AS DRUG DELIVERY SYSTEMS TO INCREASE ANDROGRAPHOLIDE BBB PERMEABILITY

INTRODUCTION

The major hindrance in the treatment of brain disorders is the blood–brain barrier (BBB), which prevents the transfer of most drugs, peptides and large molecules across the endothelial cell lining to protect the brain from undesirable side effects. To overcome such problems various approaches are used.

The liposomes offer a promising tool to resolve the low permeability and high selectivity of the BBB.

Liposomes are non-toxic, biocompatible and biodegradable drug carrier systems. Their structure which is composed of phospholipids with an aqueous reservoir allows the encapsulation of a wide variety of hydrophilic and hydrophobic agents (del Burgo et al., 2014; Pattni et al., 2015; Bilia et al., 2016). Their phospholipid bilayer structure, similar to physiological membranes, makes them more compatible with the lipid layer of BBB and increase the permeability of the drug.

Liposomes allow relatively higher intracellular uptake than other particulate systems, due to their sub-cellular size. They are highly studied for the treatment of central nervous system's pathologies such as infections, cerebral ischemia, brain tumours and neurodegenerative diseases, for instance, Parkinson's and Alzheimer's (Agrawal et al., 2017; E. Conti et al., 2017). Several studies have reported an increased transport across the BBB of encapsulated drugs both through intracerebral and intravenous administration (Garcia-Garcia et al., 2015). The surface can be modified with functional ligands to enhance the brain targeting.

The functionalized nanoparticles with structures able to interact with targets on the surface of the BBB represents a tool of enormous potentiality to ameliorate the bioavailability and to reduce side effects. Several studies on animal models of Alzheimer's disease demonstrated the efficacy of functionalized liposomes to cross BBB and ameliorate impaired cognitions (Balducci et al., 2014; Ordonez-Gutierrez et al., 2015; Mancini et al., 2016).

In previous research studies, solid lipid nanoparticles (Graverini, Piazzini et al., 2018) and polymeric nanoparticles (Guccione et al., 2017) to deliver the andrographolide (AG) through the central nervous system and ameliorate its biopharmaceutical characteristics.

The high lipid solubility of AG would permit its penetration of the BBB but its poor water solubility and stability reduces its bioavailability: indeed, these factors are the most significant drawbacks for clinical application (M. Wang et al., 2009).

In recent years, surfactants such as Tween 80 have been studied for the application in liposomal formulations. The sterically stabilized liposomes exhibited a superior entrapment stability compared with surfactant-free liposomes (Crosasso et al., 2000). The surfactant during the preparation of liposomes helps in efficient emulsification resulting in decreasing the size of vesicles and promotes the flexibility of the vesicle to penetrate the biological cell membranes.

Tween 80 was also able to enhance liposomes half-life (Bucke et al., 1998) and, in addition, has interesting properties including the formation of a superficial coating on liposomes that can produce “stealth” nanocarriers. Tween 80 can adsorb ApoE, which subsequently binds to its specific LDL receptor by increasing carrier endocytosis at the level of cerebral endothelial cells (Gulyaev et al., 1999; Kreuter et al., 2002; Wohlfart et al., 2012; Kreuter 2013; Grossi et al., 2017). Finally, this surfactant is also an inhibitor of the P-gp effluent pump (Gastaldi et al., 2014).

Another approach is the use of the cationic liposomes, able to cross the BBB via absorption-mediated transcytosis (Wong et al., 2012). Several studies have shown that these cationic nanocarriers are more efficient vehicles for drug delivery to the brain than conventional, neutral, or anionic liposomes, possibly due to the electrostatic interactions between the cationic liposomes and the negatively charged cell membranes, enhancing nanoparticle uptake. In particular, this kind of liposome interacts with the endothelial cells of microvessels rich in lecithin, which binds positively charged material and induces its cell internalization process through endocytosis (Wong et al., 2012; Agrawal et al., 2017). Furthermore, the cationic liposomes very easily fuse with cells.

The aim of the present study was the formulation of nano-sized liposomes of AG for brain targeting. Tween 80 alone or in combination with Didecyldimethylammonium bromide (DDAB) were considered to investigate the effects, on chemical and physical aspects, stability, release characteristics, *in vitro* uptake and permeability of the AG liposomes and to ameliorate the loading and the solubility of AG.

Liposomes were evaluated for various formulation parameters (size, polydispersity, ζ -potential, morphology, chemical and physical stability, *in vitro* release) and the optimized formulations were studied and characterized by *in vitro* tests.

The ability of liposomes to increase the permeability of AG was evaluated by a Parallel Artificial Membrane Permeability Assay (PAMPA) (Di et al., 2003). Furthermore, the uptake of liposomes as well as their permeability across hCMEC/D3 monolayer cells, as an *in vitro* BBB model (Eigenmann et al., 2013; Guccione et al., 2017), were considered. Cell viability and cytotoxicity studies were also conducted.

MATERIALS

Egg phosphatidylcholine (Phospholipon 90G) was purchased from Lipoid AG, Cologne, Germany with the support of its Italian agent AVG srl, Milan, Italy. Andrographolide, Cholesterol $\geq 95\%$, Didecyldimethylammonium bromide (DDAB, 98%), Coumarin-6 (6C), Fluorescein sodium salt (NaF), Human Serum Albumin (HSA), Phosphate Buffered Saline (PBS 0.01 M) powder (29 mM NaCl, 2.5 mM KCl, 7.4 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.3 mM KH_2PO_4) pH 7.4 and Tween 80 (Polyethylene glycol sorbitan monooleate) were from Sigma Aldrich, Milan, Italy. Glucose anhydrous and sucrose came from Merck (Darmstadt, Germany). 96-well Multi-Screen PAMPA filter plate (pore size 0.45 μm) were purchased from Millipore Corporation (Tullagreen, Carrigtwohill, County Cork, Ireland). Porcine polar brain lipid was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All the solvents used (acetonitrile, dichloromethane, dodecane, ethanol, formic acid, methanol) were from Sigma Aldrich (Milan, Italy). Water was purified by Millipore (Milford, MA, USA) Milli-Q_{plus} system. Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, PA, USA).

METHODS

Preparation of Liposomal Carriers

Stealth liposomes containing Tween 80 (LPs) and cationic liposomes (CLPs) with Tween 80 and DDAB were prepared according to the thin layer evaporation method (Bangham et al., 1974). For LPs, 160 mg of egg phosphatidylcholine (P90G) and 10 mg of cholesterol (CHOL) were dissolved in dichloromethane. The organic solvent was vacuum evaporated, and the dry lipid film was hydrated by adding 10 mL PBS containing Tween 80 at a concentration of 3% w/v. The aqueous dispersion was shaken with a mechanical stirrer for 30 min in a water bath at the constant temperature of 37 °C. In order to obtain small unilamellar vesicles from multilamellar vesicles, an ultrasonication probe was used for 10 min (with pulsed duty cycles of ½ s on and ½ s off, amplitude 50%) with the sample in an ice bath to prevent lipid degradation (Righeschi et al., 2014). Finally, gentle centrifugation of 1 min at $1205 \times g$ was performed to remove possible metallic particles released by the ultrasonic probe inside the liposomal dispersion.

In addition, for CLPs, 10 mg of DDAB were weighted together with P90G and CHOL and then vesicles were prepared by hydrating the dry lipid film with 10 mL PBS containing 3% of Tween 80 (Saengkrit et al., 2014).

AG-loaded LPs (LPs-AG) and AG-loaded CLPs (CLPs-AG) were prepared with the same method described above, adding 8.5 mg of AG (0.85 mg/mL, corresponding to 5% of the weight of the lipid component) together with P90G, CHOL, DDAB in the case of CLPs-AG and 1–2 mL of methanol with dichloromethane to completely dissolve AG.

Coumarin-6-loaded liposomes (LPs-6C and CLPs-6C) were prepared using the same method, adding 5 mg of the probe ($\lambda_{\text{max}} = 444$, $\lambda_{\text{ex}} = 420$ nm, $\lambda_{\text{em}} = 505$ nm, green), corresponding to 3% of the weight of the lipid component, to the organic phase.

Physical and Morphological Characterization

Liposomes' hydrodynamic diameter, size distribution and ζ -potential were measured by Light Scattering (LS), using a Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK) outfitted with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25 °C. Time correlation functions were analyzed by the Cumulant method, to obtain the hydrodynamic diameter of the vesicles (Z_{average}) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. ζ -potential, instead, was calculated from the electrophoretic mobility, using the Henry correction to Smoluchowski's equation. The samples were diluted 100-fold in distilled water and an average of three measurements at stationary level was taken. A Haake temperature controller kept the temperature constant at 25 °C.

Liposomes were also analyzed concerning morphology, shape, and dimensions by the transmission electron microscopy (TEM). The aqueous dispersion was diluted 10-fold in PBS and 5 μL were applied to a carbon film-covered copper grid. Most of the sample was blotted from the grid with filter paper to form a thin film. After the adhesion of liposomes, 5 μL of phosphotungstic acid solution (1% w/v in sterile water) were dropped onto the grid as a staining medium and the excess solution was removed with filter paper. Samples were dried for 3 min, after which they were examined with a JEOL 1010 electron microscope (Tokyo, Japan) and then photographed at an accelerating voltage of 64 kV.

Chemical Characterization of Formulations

The percentage of the AG or 6C entrapped into liposomes in respect to the amount of substances initially used in the liposomal preparation was expressed as encapsulation efficiency (EE%) and calculated using the direct method. Free AG or 6C was removed by means of dialysis. 2 mL of liposomal suspensions were transferred in a dialysis bag (cut-off 3500–5000 Dalton), which was stirred in 1 L of water at room temperature for 1 h (Righeschi et al., 2014). The content of AG or 6C entrapped within liposomes was quantified by HPLC-DAD analysis, respectively after disruption with methanol of purified liposomes (placed in the ultrasonic bath for 30 min) and ultracentrifugation for 10 min at $11,330 \times g$.

LC% for liposomal formulations was calculated using the following equation:

$$\text{LC\%} = \frac{\text{Total amount of determined drug}}{\text{Weight of liposomes}} \times 100$$

The Recovery% was carried out with the same procedure but without initial dialysis and was calculated using the following formula:

$$\text{Recovery\%} = \frac{\text{Total amount of determined drug}}{\text{Initial amount of drug loading}} \times 100$$

HPLC-DAD and HPLC-FLD Methods

An HP 1100 liquid chromatograph equipped with a DAD detector was used to carry out the quali-quantitative determinations of AG. A 150 mm × 4.6 mm i.d., 5 µm Zorbax Eclipse XDB, RP18 column (Agilent Technologies, Santa Clara, CA, USA) was employed. The mobile phases were (A) CH₃CN and (B) formic acid/water pH 3.2. Flow rate was 0.8 mL/min and temperature were set to 27 °C. The following gradient profile was utilized: 0–2 min, 5–15% A, 95–85% B; 2–5 min, 15% A, 85% B; 5–7 min 15–50% A, 85–50% B; 7–12 min, 50% A, 50% B; 12–15 min, 50–30% A, 50–70% B; 15–20 min, 30% A, 70% B; 20–25 min, 30–5% A, 70–95% B with equilibration time of 5 min. The UV/vis spectra were recorded in the range 200–800 nm and the chromatograms were acquired at 223 nm.

6C characterization was performed using an HP 1200 liquid chromatograph with Luna RP18 column (4.6 mm × 250 mm i.d., 5 µm) maintained at 25 °C (All from Agilent Technologies, Santa Clara, CA, USA). The mobile phase was composed of (A) CH₃CN and (B) formic acid/water pH 3.2 with a flow rate of 1 mL/min. The gradient profile was: 0–2 min, 30% A, 70% B; 2–26 min 30–100% A, 70–0% B; 26–29 min 100% A, 0% B; 29–35 min 100–30% A, 0–70% B with post-time of 5 min. Chromatograms were acquired at 444 nm.

An HP 1200 liquid chromatograph equipped with an FLD detector was used for the quantification of NaF probe ($\lambda_{\text{ex}} = 460$ nm, $\lambda_{\text{em}} = 515$ nm, green). The column was a Kinetex C18 (4.6 mm × 150 mm i.d., 5 µm) maintained at 27 °C (All from Agilent Technologies, Santa Clara, CA, USA). The mobile phases were (A) CH₃CN and (B) formic acid/water pH 3.2. Flow rate was 0.8 mL/min. The following gradient profile was utilized: 0–3 min, 20% A, 80% B; 3–23 min, 20–80% A, 80–20% B; 23–25 min 80–100% A, 20–0% B; 25–27 min, 100–20% A, 0–80% B with equilibration time of 5 min.

Diluting stock solutions in CH₃OH (0.5 mg/mL for AG and 0.1 mg/mL for 6C) and in H₂O (0.1 mg/mL for NaF), standard solutions were freshly prepared. To quantify each compound, an external standard method was applied using a regression curve and analyses were performed in triplicate. Results were expressed as the mean $\pm$ SD of the 3 experiments.

All the compounds showed a linear response: AG from 0.05 to 25 μ g/mL, NaF from 0.05 to 46 μ g/mL and 6C from 0.515 to 51.5 μ g/mL. All the curves had coefficients of linear correlation $R^2 \geq 0.999$.

Progressive dilutions of standard solutions were used to calculate the limit of detection LOD ($S/N \geq 3$) and the limit of quantification LOQ ($S/N \geq 10$). LOD and LOQ for AG were 2.6 ng and 5.3 ng, respectively.

Stability Studies

The stability of empty and AG-loaded liposomes was studied for one month. Aqueous dispersions were kept at 4 °C and, at fixed time intervals, their physical and chemical stabilities were assayed: physical stability was checked by monitoring sizes, polydispersity index and ζ -potential, while chemical stability was determined by quantification of the encapsulated drug by HPLC-DAD analysis.

The freeze-drying process in the absence of cryoprotectant and in the presence of 1% *w/v* of glucose or sucrose was also considered. Afterwards, lyophilization physical stability was checked for one month at 25 °C.

200 μ L of LPs and CLPs dispersions were incubated at body temperature with a solution of human serum albumin (HSA, 40 mg/mL in PBS) for two hours under magnetic stirring to mimic *in vivo* conditions (Gualbert et al., 2003; Koziara et al., 2004). Physical stability of the formulations was evaluated using Dynamic Light Scattering, by controlling liposomes sizes at regular intervals.

The yield of the preparation of freeze-dried LPs-AG and CLPs-AG was calculated as the weight of the product obtained after the freeze-drying, compared to the weight of the components used in the reaction:

$$\text{Yield\%} = \frac{\text{real weight (mg)}}{\text{teoric weight (mg)}} \times 100$$

In Vitro Release

AG *in vitro* release from liposomes was performed using a dialysis membrane (cut-off 3000–5000 Dalton) in PBS at 37 °C. Two mL of AG solution (0.85 mg/mL in methanol), LPs and CLPs suspensions were filled in pre-soaked dialysis tubes and placed in 200 mL of release medium using a magnetic stirrer. An aliquot of 1 mL of release medium was removed at pre-determined time intervals and replaced with 1 mL of fresh PBS maintained at 37 °C (Righeschi et al., 2016). AG concentration at different times was calculated using HPLC analyses: the mean of triplicate drug release and standard deviation (mean ± SD, n = 3) was used to draw the drug release profiles.

The following equation was applied to calculate the percentage of AG released in the medium at pH 7.4 at each time interval (0, 30, 60, 120, 240, 360 and 1440 min):

$$\% \text{ drug released} = \frac{\text{drug(t)(mg)}}{\text{total drug (mg)}} \times 100$$

To evaluate the kinetics and mechanism of drug release from the liposomes, the Korsmeyer–Peppas model, Hixson Crowell model, Higuchi model, first order and zero order mathematical models were used and the best-fitted model was selected based on high regression coefficient (R²) value for the release data.

PAMPA Studies

PAMPA studies for LPs-AG and CLPs-AG were carried out using the method previously reported. A solution (2% *w/v*) of Porcine Polar Brain Lipid (PBL) in *n*-dodecane was prepped and the mixture was sonicated. PBL solution (5 μ L) was added to each donor plate well. Right after the application of the artificial membrane, 250 μ L of the formulation were added to each donor compartment, whilst the acceptor compartment was filled with PBS/Ethanol solution. Then the drug-filled donor compartment was installed into the acceptor plate. After incubation for 18 h, the donor and acceptor plate samples were withdrawn and analyzed by HPLC-DAD analyses for quantification of AG concentration: 150 μ L were taken from both compartments, later diluted with methanol, placed in the ultrasonic bath for 30 min and finally ultra-centrifuged for 10 min at 11,330 $\times$ *g* (4 $^{\circ}$ C). The permeability of AG was calculated using the following formula (Wohnsland & Faller, 2001):

$$P_e = -\ln [1 - C_A(t)/C_{\text{equilibrium}}]/A \times (1/V_D + 1/V_A) \times t$$

where P_e is permeability in the unit of cm/s, effective filter area (A) = $f \times 0.3 \text{ cm}^2$, where f = apparent porosity of the filter, $C_A(t)$ = compound concentration in receptor well at time t , V_D = donor well volume (mL), V_A = receptor well volume (mL), t = incubation time (s), $C_D(t)$ = compound concentration in donor well at time t , and:

$$C_{\text{equilibrium}} = [C_D(t) \times V_D + C_A(t) \times V_A]/(V_D + V_A)$$

The experiments were performed in triplicate.

hCMEC/D3 Cell Culture

This cell line (Millipore Cat. # SCC066) derives from human temporal lobe microvessels isolated from tissue excised during surgery for epilepsy control. Cells were seeded in a concentration of 2.5×10^4 cells/cm² and grown at 37 °C in an atmosphere of 5% CO₂ in 25 cm² rat tail collagen type I coated culture flasks. EndoGRO™-MV Complete Media Kit (Cat. # SCME004) supplemented with 1 ng/mL FGF-2 (Cat. #GF003) was changed every three days and cells were grown until they were 90% confluent. Cells were passaged at least twice before use. Confluent hCMEC/D3 cells were split by Accumax™ Cell Counting Solution in DPBS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

To assess cell viability after AG and LPs-AG and CLPs-AG exposure, an MTT assay was performed (Conti et al., 2010; Landucci et al., 2016). Cells were seeded in a 24-well plate (6×10^4 cells/cm²) pre-coated with Collagen Type I, Rat Tail (Cat. #08–115) and grown at 37 °C in an atmosphere of 5% CO₂ in EndoGRO™ Basal Medium (EBM-2). When the cells were approximately 70–80% confluent they were incubated with different concentrations of AG (10 and 100 µM), LPs-AG (0.085 and 0.0085 mg/mL) and CLPs-AG (0.085 and 0.0085 mg/mL), obtained by dilution (1:10 and 1:100) of the formulation in EBM-2 for 2, 4 and 24 h. The liposome formulations were previously filtered through 0.4 µm sterile filter units. The medium of each well was separated from the cells and stored for lactate dehydrogenase (LDH) assay, and cells were treated with 1 mg/mL of MTT for 1 h at 37 °C and 5% CO₂. Finally, DMSO was added to dissolve MTT formation and absorbance was measured at 550 and 690 nm. Cell viability was expressed as a percentage compared to the cells incubated only with EBM-2 (positive control). Triton X-100 was used in the MTT assay as the negative control since its detergent action disrupts the cells.

LDH Assay

Cytotoxicity after AG and liposomes exposure was verified with LDH assay. The medium resulting from incubation of AG and liposomes with cells was centrifuged (250 g, 10 min at RT) and the supernatant separated from the deposited cells in each well. This centrifugation process allowed us to remove any waste and cellular debris as well as AG and liposomes. The release of LDH into culture supernatants was detected by adding the catalyst and dye solutions of a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, IN, USA). The absorbance values were recorded at 490 nm and 690 nm. Cytotoxicity was expressed as a percentage compared to the maximum LDH release in the presence of triton X-100 (positive control). EBM-2 was used as negative control since no cytotoxicity was detected in such conditions.

hCMEC/D3 Cell Culture for Transwell Permeability Studies

High-density pore (2×10^6 pores/cm²) transparent PET membrane filter inserts (0.4 μ m, 23.1 mm diameter, Falcon, Corning BV, Amsterdam, Netherlands) were used in 6-well cell culture plates (Falcon, Corning, Amsterdam, Netherlands) for all transcytosis assays. The transparent PET membrane filter inserts were coated with rat tail collagen type I at a concentration of 0.1 mg/mL and incubated at 37°C for 1 h before cell barrier coating. Inserts were subsequently washed with PBS and incubated for 1 h, after which PBS was removed and replaced with the assay medium. The inserts were calibrated for at least 1 h with assay medium at 37°C. Optimum media volumes were calculated to be 1 mL and 1.2 mL respectively for the apical and basolateral chambers. The transwell inserts were calibrated with assay medium for 1 h, then the medium was removed and hCMEC/D3 cells were seeded onto the apical side of the inserts at a density of 6×10^4 cells/cm² in 1 mL assay media. 1.2 mL of fresh medium was added to the basolateral chamber.

The assay medium was changed every 3 days following transwell apical insert seeding with hCMEC/D3. For seven days, cells were grown to confluence. hCMEC/D3 monolayers were used as a permeability assay for AG and AG-loaded liposomes. Fluorescein sodium salt (NaF) was considered at a concentration of 10 $\mu\text{g/mL}$ as an integrity control marker with a known permeability coefficient (P_{app}) for this cell line (Gulyaev et al., 1999). The integrity of monolayer cells was also confirmed by observation of cultures under phase-contrast microscopy or bright-field optics using of transparent membranes. The image was observed using an inverted microscope (Olympus IX-50; Solent Scientific, Segensworth, Fareham, UK) with a low-power objective (20X). The images were digitized using a video image obtained with a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA).

For permeability studies, AG (10, and 100 μM), LPs-AG and CLPs-AG (0.085 mg/mL, corresponding to AG 240 μM) obtained by dilution 1:10 of the formulation in EBM-2 were tested and incubated for 1, 2, 3 and 4 h in the apical donor compartment. At the end of the incubation, the amount of NaF and AG were quantified both in apical and basolateral compartments by HPLC-FLD or HPLC-DAD method. In the case of the formulation, EBM-2 was diluted with methanol and placed in the ultrasonic bath for 30 min and then ultra-centrifuged for 1 h at $11,330\times g$ (4 °C).

The apparent permeability coefficients (P_{app}) of free AG and AG encapsulated in LPS and CLPs were calculated according to the equation:

$$P_{app} \text{ (cm/s)} = V_D / (A M_D) \times (\Delta M_R / \Delta t)$$

where: V_D = apical (donor) volume (cm^3), M_D = apical (donor) amount (mol), $\Delta M_R / \Delta t$ = change in amount (mol) of compound in receiver compartment over time.

The recovery for AG and NaF was calculated according to the equation:

$$\text{Recovery (\%)} = C_{Df} V_D + C_{Rf} V_R / (C_{D0} V_D) \times 100$$

where C_{Df} and C_{Rf} are the final compound concentrations in the donor and receiver compartments, C_{D0} is the initial concentration in the donor compartment and V_D and V_R are the volumes in the donor and receiver compartments, respectively. All experiments were performed at least in triplicate.

Cellular Uptake of LPs-6C and CLPs-6C

For the evaluation of the intracellular content of 6-Coumarin, hCMEC/D3 cells (1×10^4) were exposed for 2 h to the LPs-6C and CLPs-6C loaded with 0.5 mg/mL of 6C and diluted 1:100 into EBM-2, and to a saturated solution of fluorescent probe. To elucidate the endocytic uptake mechanisms, these experiments were carried out in presence/absence of endocytic inhibitors. Control cells were exposed to liposomal formulations without any agent pre-treatment and their uptake was assumed to be 100%. A second group of cells was pre-treated with 15 μM chlorpromazine for 30 min followed by incubation with liposomes. A third group of cells was pre-treated with 25 μM of indomethacin for 30 min; and, finally, a fourth group of cells was maintained at 4 °C during the LPs-6C and CLPs-6C exposure to observe the effect of low temperature, a general metabolic inhibitor.

At the end of the treatments, the amount of 6C was quantified on cellular lysate by HPLC. For control cells and cells maintained at 4 °C during exposure, a morphological evaluation of cellular uptake was also performed: hCMEC/D3 cells were cultured on histological slides, treated as described above, fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 10 min then stained with Fluoro scheld with DAPI (Sigma, Milan, Italy) to display the nucleus and observed by fluorescence microscopy (Labophot-2 Nikon, Tokyo, Japan). Ten photomicrographs were randomly taken for each sample.

Cellular uptake was investigated by confocal microscopy Nikon Eclipse Ti using liposomes labelled with 6C, with S Fluor 20x, NA = 0.75 high pressure Hg vapor lamp (Intensilight, Nikon, Tokyo, Japan).

Filter set: excitation 365 nm emission 400 nm hi-pass DAPI, excitation 485 nm emission 524 nm 6Co and CCD camera: Coolsnap HQ², Princeton Instruments, Trenton, NJ, USA, 1392 × 1040, 6.45 um square pixels.

Statistical Analysis

The experiments were repeated three times and results expressed as a mean ± standard deviation. Statistical significance of hCMEC/D3 cell viability and cellular uptake was analyzed using one-way ANOVA followed by the post hoc Tukey's w-test for multiple comparisons. All statistical calculations were performed using GRAPH-PAD PRISM v. 5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value p of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Liposomes

LPs were prepared by using P90G, CHOL and Tween 80. This compound was selected as a coating agent to increase the stability of the formulation, to produce “stealth” nanovesicles and to promote endocytosis of the carrier at the level of cerebral endothelial cells (Gastaldi et al., 2014). Various ratios of the two lipid constituents were tested to obtain small sizes, good polydispersity and favourable ζ -potential. In particular, the ratios P90G:CHOL 18:1, 16:1, 14:1, 12:1, and 10:1 were considered. The best ratio resulted to be 16:1, corresponding to 160 mg of P90G and 10 mg of CHOL. Then, 3% w/v of Tween 80 was added (LPs). Furthermore, different sonication times were tested to optimize LPs physical characteristics. The selected conditions consisted in two cycles of 5 min of sonication, each including 0.5 s of sonication alternating with 0.5 s of pause, as reported in the materials and methods section. LPs were nanosized unilamellar vesicles, with a PDI less than 0.25 and a ζ -potential, around -20 mV (Table 1), confirming a homogeneous and stable dispersion. LC% was $2.28\% \pm 0.22$. The mean vesicle sizes and the width of the particle distribution are important parameters as they govern physical stability and permeation through BBB (Chavan et al., 2013). Moreover, the vesicles sizes highly affected the interaction of the liposomes with the hCMEC/D3 cellular model (Markoutsas et al., 2011; Papadia et al., 2016).

AG does not influence the stability of the formulation (Table 1); when LPs were loaded with AG, LPs-AG showed the same physical parameters as LPs. The electron microscope analysis confirmed the liposomal structure; the results evidenced the presence of spherical vesicles, with a defined phospholipid bilayer, well separated, due to the presence of the surfactant that prevents agglomeration, and with dimensions around 100 nm, confirming the DLS results (Figure 1a).

Next, LPs were functionalized with positive surface charges by using DDAB (cationic liposomes, CLPs) in the same amount of CHOL (10 mg in the total formulation). In this case, ζ -potential resulted positive, indicating the presence of positive charges on the surface of the carrier.

Then, CLPs loaded with 8.5 mg of AG (corresponding to 5% of the weight of the lipid component) were prepared (CLPs-AG); the presence of DDAB and AG did not modify the physical characteristics of the formulation (Table 1). LC% was $3.08\% \pm 0.21$. TEM analysis showed well separated spherical shape vesicles, with a distinct phospholipid bilayer (Figure 1b).

Sample	Size (nm)	PdI	ζ -Potential	EE%	Recovery%
LPs	80.2 ± 3.6	0.22 ± 0.03	-20.4 ± 4.1	-	-
CLPs	84.6 ± 8.1	0.23 ± 0.02	20.7 ± 4.7	-	-
LPs-AG	96.4 ± 9.5	0.23 ± 0.03	-22.8 ± 1.2	44.7 ± 3.2	91.1 ± 5.3
CLPs-AG	82.1 ± 9.3	0.25 ± 0.01	20.3 ± 3.7	47.5 ± 3.3	94.9 ± 4.7
LPs-6C	193.1 ± 3.0	0.21 ± 0.02	-27.4 ± 0.4	46.0 ± 1.4	71.2 ± 4.2
CLPs-6C	197.1 ± 1.4	0.27 ± 0.03	31.1 ± 0.6	63.1 ± 0.1	80.6 ± 5.0

Table 1. Physical characterization of empty, andrographolide (AG) and coumarin-6 (6C) loaded liposomes.

Data displayed as mean $\pm$ SD; n = 3.

The compound 6-Coumarin (6C), a lipophilic fluorescent dye, was incorporated into liposomes to investigate the ability of nanoparticles to penetrate into hCMEC/D3 cells, as BBB-model, and to elucidate trans-endothelial transport *in vitro*. The preparation of fluorescent liposomes was performed as reported for LPs and CLPs, by adding 6C (0.5 mg/mL) to the organic phase. Their physical and chemical parameters are shown in Table 1. The same dimensions of the two types of liposomes, was very important to interact with the hCMEC/D3 equally (Papadia et al., 2016). LPs-6C and CLPs-6C resulted larger but useful for a parenteral administration. AG and 6C are lipophilic compounds and there are inserted in the bilayer, but the effect on the sizes of nanoparticles is different due to their unlike chemical structure. The high ζ -potential of all formulations is indicative of their stability, as also supported by stability studies.

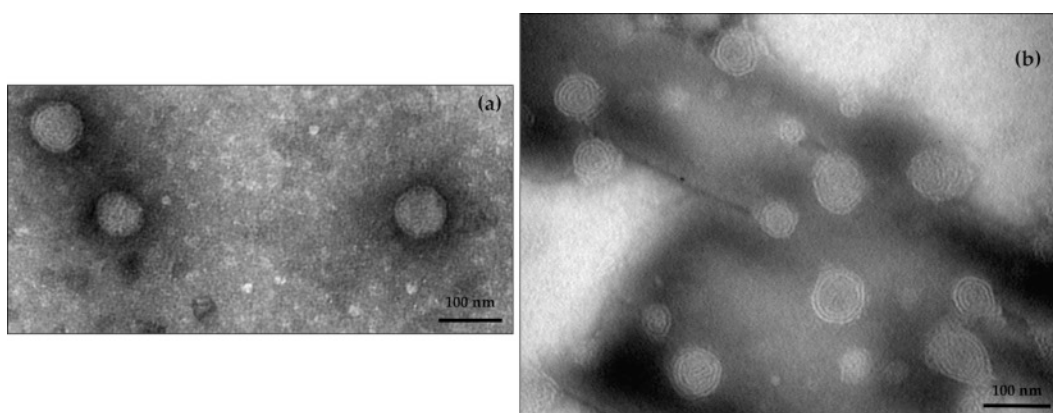


Figure 1. TEM images of LPs-AG (a) and CLPs-AG (b).

Scale 100 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Stability Studies

Liposomes stability was evaluated both as a colloidal dispersion and in the freeze-dried form. The ability of the aqueous dispersions to maintain their physicochemical properties concerning particle size, Pdl, surface charge and drug entrapment was assessed after 1-month storage at 4 °C and the Light Scattering analyses were performed to control the stability over time. No significant changes were observed in physical parameters of empty or LPs-AG and CLPs-AG dispersions (Figure 2). The presence of non-ionic surfactant is expected to reduce the agglomeration between liposomes via steric repulsion.

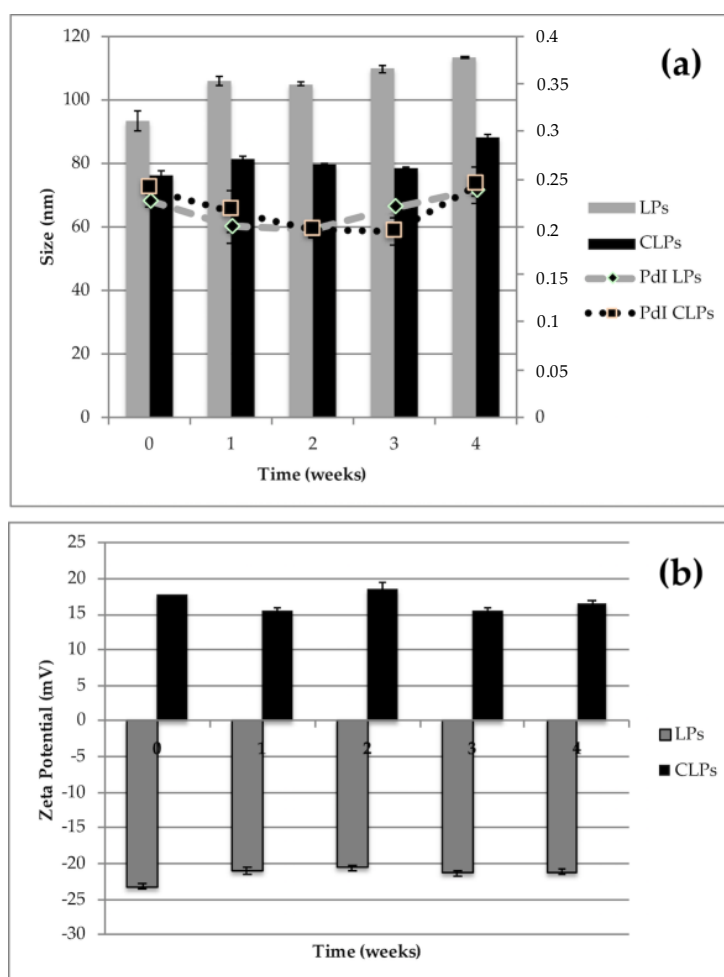


Figure 2. Particle size, polydispersity index (PdI) (a) and ζ -potential (b) of LPs-AG and CLPs-AG as dispersion after one-month storage at 4 °C.

Data displayed as mean $\pm$ SD; n = 3.

Also, the presence of DDAB on the surface of liposomes prevented the aggregation and the precipitation of the vesicles and increased the system's stability. In addition, the entrapment efficiency remained constant, around 45%. The major limitation to liposomes use is due to their physical and chemical instability, when the aqueous suspension is stored for an extended period. The poor stability in an aqueous medium forms a real obstacle against the clinical application of nanoparticles.

To improve the physical and chemical stability, water needs to be removed. Freeze-drying is a good technique to enhance the chemical and physical stability of formulations over prolonged periods.

The stability of LPs-AG and CLPs-AG with time was also evaluated in the freeze-dried form. However, the freezing process of the sample might cause problems of possible structural and/or functional damages of the system, and/or subsequent difficulties in sample re-solubilization, due to particle aggregation phenomena. The addition of cryoprotectants improves the quality of the dehydrated product, decreases particle aggregation phenomena and allows to obtain an easier re-dispersion of the freeze-dried product.

Therefore, to estimate the effect of the presence and type of cryoprotectant, empty, LPs-AG and CLPs-AG formulations were freeze-dried with and without sucrose or glucose (1% w/v). After the lyophilization process, all formulations were dispersed in PBS and analyzed by DLS and ELS (Table 2). As shown in Table 2, the drying process produced an increase in terms of size and PdI, respect to the values reported before the freeze-drying process (Table 1). However, all liposomes maintained characteristics suitable for parenteral administration. The best freeze-drying process was obtained in the presence of glucose both for LPs-AG and CLPs-AG. The EE% remained almost constant around 43%. The yield % of the preparation process was also calculated, in this case without addition of the cryoprotectant, and resulted $69.5\% \pm 0.1$ for LPs and $71.2\% \pm 0.1$ for CLPs

(mean $\pm$ SD; $n = 3$). After a month of storage at 25 °C the freeze-dried product retained the starting characteristics.

A drawback to the use of nanocarriers, in particular cationic nanocarriers, for brain delivery is their binding to serum proteins that attenuates their surface charge. Therefore, the stability of LPs-AG and CLPs-AG in the presence of human serum albumin (HSA) at physiological concentration was also tested. After 2 h of incubation, DLS analyses confirmed that sizes were not affected by the presence of HSA and therefore revealed coexistence of free serum proteins and optimized nanocarrier without any protein corona effect (Table 3).

LPs-AG	No Cryoprotector	Glucose	Sucrose
Size (nm)	148.8 $\pm$ 1.4	135.0 $\pm$ 0.9	147.5 $\pm$ 1.2
PdI	0.32 $\pm$ 0.03	0.25 $\pm$ 0.02	0.35 $\pm$ 0.01
ζ (mV)	-21.3 $\pm$ 0.9	-19.4 $\pm$ 1.1	-18.5 $\pm$ 1.0
CLPs-AG			
Size (nm)	144.6 $\pm$ 2.2	131.3 $\pm$ 5.1	149.3 $\pm$ 1.2
PdI	0.38 $\pm$ 0.02	0.28 $\pm$ 0.01	0.29 $\pm$ 0.01
ζ (mV)	+28.6 $\pm$ 0.9	+27.0 $\pm$ 0.8	+26.5 $\pm$ 0.9

Table 2. Physical parameters of AG-loaded liposomes, after the freeze-drying process with and without cryoprotectant, 1% *w/v* of sucrose or glucose.

Data displayed as mean $\pm$ SD; $n = 3$.

Time	LPs-AG		CLPs-AG	
	Size (nm)	Pd	Size (nm)	Pd
0	94.8 $\pm$ 2.4	0.23 $\pm$ 0.02	76.4 $\pm$ 1.2	0.24 $\pm$ 0.01
30'	103.8 $\pm$ 2.0	0.39 $\pm$ 0.01	82.9 $\pm$ 0.5	0.41 $\pm$ 0.02
1 h	97.2 $\pm$ 3.2	0.39 $\pm$ 0.02	83.5 $\pm$ 4.8	0.40 $\pm$ 0.01
2 h	99.1 $\pm$ 5.1	0.39 $\pm$ 0.01	81.9 $\pm$ 3.4	0.43 $\pm$ 0.02

Table 3. Physical stability of AG-loaded liposomes in presence of human serum albumin.

Data displayed as mean $\pm$ SD; $n = 3$.

In Vitro Release

AG *in vitro* release at 37 °C from LPs-AG and CLPs-AG was evaluated for 24 h by using a dialysis bag and PBS as receptor medium to mimic sink conditions. The release profiles of AG from AG solution, LPs-AG and CLPs-AG were reported in Figure 3. The result indicated that the release of AG from methanol solution through the dialysis membrane was much faster, with a fast release during the first 2 h and approximately 100% of the drug released within 6 h. In contrast, the immediate release of the drug (burst effect) does not occur in the case of LPs-AG and CLPs-AG. The percentages of AG released from LPs and CLPs were gradual: only 56.8% and 69.7% of the drug were released within 6 h, respectively. The percentages rose to 83.5% and 77.4%, after 24 h, respectively. The almost linear and gradual trend of the release indicated that the liposomal systems can release AG for prolonged periods and in greater quantities compared to the saturated aqueous solution, where the solubility of AG resulted very low (0.05 mg/mL). Optimized liposomal formulations are able to solubilize 0.85 mg/mL of drug.

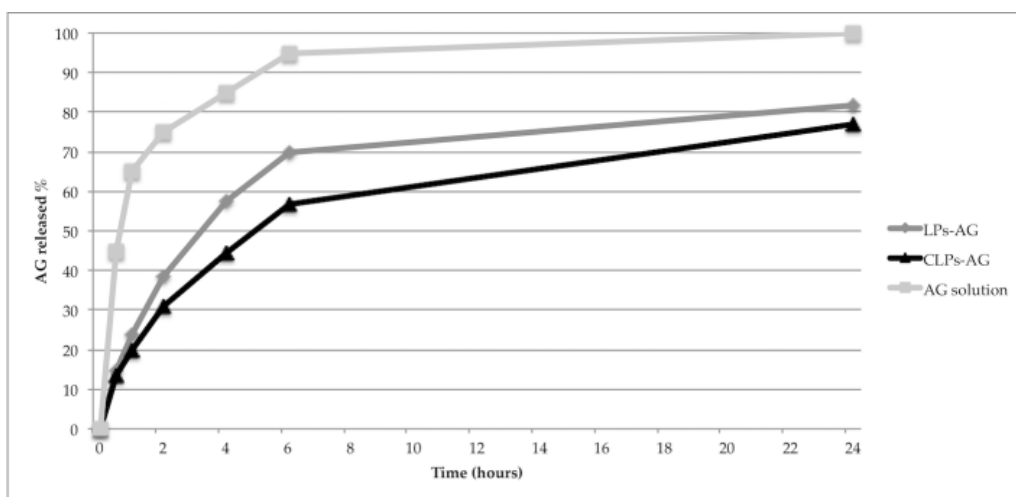


Figure 3. *In vitro* release profiles of LPs-AG and CLPs-AG in PBS.
Each data point represents the average of three samples.

Different theoretical models were considered to examine the nature of the release. The drug release mechanism was defined by fitting AG release data with various kinetics models. By comparing the regression coefficient values, the Higuchi model ($R^2 = 0.8366$ and 0.9264 , respectively, Table 4) resulted as the best to describe the kinetics of these two types of liposomes. Thus, the liposomal membrane disruption controlled the release mechanism (Mohan et al., 2016).

Due to the very low solubility of AG in water and the related problems of bioavailability, both the liposomal formulations allow the administration of a high amount of solubilized molecule according to the requirements for parenteral preparations.

Release Kinetics	LPs-AG	CLPs-AG
Zero order	0.5722	0.7079
First order	0.7685	0.8816
Korsmeyer-Peppas	0.4552	0.4980
Hixson	0.7033	0.8292
Higuchi	0.8366	0.9264

Table 4. Regression coefficient (R^2) obtained in different kinetics models for AG release from LPs-AG and CLPs-AG.

PAMPA Study

Parallel Artificial Membrane Permeability Assay (PAMPA) was performed to estimate passive transcellular permeability. It is a non-cell-based permeability model because it lacks transporter- and pore-mediated permeability, but is considered robust, reproducible and it results in a helpful complement to the cellular permeability model for its speed, low cost and versatility, and readily provides information about passive transport permeability.

AG is a molecule with low BBB permeability (effective permeability, P_e value of $0.49 \pm 0.16 \times 10^{-6}$ cm/s) and therefore liposomal formulation could represent a useful tool to improve its permeation. P_e of AG-loaded liposomes resulted as increased, in particular $3.94 \pm 0.60 \times 10^{-6}$ cm/s for LPs-AG and $3.87 \pm 0.36 \times 10^{-6}$ cm/s for CLPs-AG. These values confirmed that LPs-AG and CLPs-AG increased the permeability of the drug, of about an order of magnitude, compared to the aqueous solution.

Though this test does not discriminate the different behaviour of the two systems because the artificial membrane fails to mimic all properties of a cell, a mechanism of permeation through the artificial membrane was hypothesized. An interaction between the phospholipid bilayer, which is a flexible system, with the lipid that covers the artificial membrane, similar to one of the mechanisms of liposome-cell interaction.

MTT and LDH Assays

MTT and LDH assays were performed in the hCMEC/D3 cell line to evaluate the effect of AG and LPs-AG and CLPs-AG in cell viability and cytotoxicity, and permeability studies were also conducted in transwell devices using the same cell line. The *in vitro* cytotoxicity of the developed LPs-AG and CLPs-AG and AG was assessed by cell viability determination and membrane integrity evaluation using the hCMEC/D3 cell line in MTT and LDH assays, respectively (Figure 4).

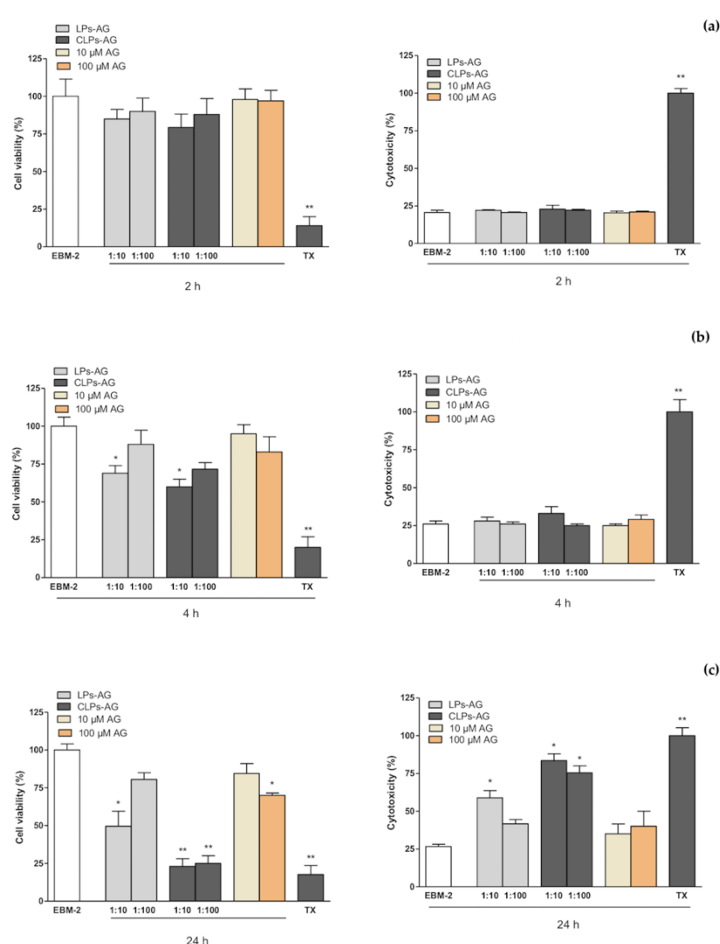


Figure 4. hCMEC/D3 cell viability evaluated by MTT assay (left panel) and cytotoxicity by LDH assay (right panel) when exposed for 2 h (a), 4 h (b) and 24 h (c) to AG (10 and 100 μM) or LPs-AG and CLPs-AG (0.0085 and 0.085 mg/mL). Data are expressed as percentage of control (EBM-2 medium) and Triton-X (TTX) which represent, respectively, the maximum cell viability and cell cytotoxicity. Values represent the mean ± SEM of at least three experiments performed in triplicate.

* $p < 0.05$ and ** $p < 0.01$ vs. EBM-2 alone.

When cells were exposed to different concentrations of AG (10 and 100 μ M) and LPs-AG and CLPs-AG (0.0085 and 0.085 mg/mL) for 2 (Figure 4a) and 4 h (Figure 4b), no significant changes were observed in MTT metabolization, except for LPs-AG and CLPs-AG (0.085 mg/mL) or LDH release when compared to cells exposed to the EBM-2 medium alone, indicating that AG and LPs-AG and CLPs-AG affected neither the metabolic activity of the cells nor the membrane integrity at these time points. On the other hand, when the cells were incubated for 24 h with AG at a dose of 100 μ M, LPs-AG (0.085 mg/mL) and CLPs-AG (0.0085 and 0.085 mg/mL) we observed a significant reduction of cell viability and an increase in cytotoxicity compared to the control (Figure 4c).

BBB Permeability Studies

hCMEC/D3 brain microvascular endothelial cell line is a model of human BBB utilized to study the drug transport mechanisms (Bucke et al., 1998; Benfer & Kissel, 2012). The cells retain the expression of most transporters and receptors expressed *in vivo* in the human BBB. hCMEC/D3 apparent permeability coefficient (P_{app}) correlates well with *in vivo* permeability data, and therefore permeability studies were performed to predict the permeability of free AG and LPs-AG and CLPs-AG across the BBB. NaF was used as the negative control and its P_{app} was determined during all transport experiments to monitor the integrity of the cell layer. This aspect was also been checked by phase-contrast microscopy. P_{app} of NaF was $8.27 \pm 1.81 \times 10^{-6}$ cm/s, in agreement with the literature values (Gulyaev et al., 1999; Guccione et al., 2017). This value remained constant during the permeability assay, and demonstrates the confluence of the monolayer and assesses the tight junction integrity. P_{app} of LPs-AG and CLPs-AG were only slightly higher than the P_{app} values of the free AG for the first 3 h. However, at 4 h, the P_{app} value for both LPs-AG and CLPs-AG was significantly higher (about double) than that of the free AG (P_{app} of AG: $8.67 \pm 0.95 \times 10^{-6}$ cm/s; P_{app} LPs-AG: $16.8 \pm 1.70 \times 10^{-6}$ cm/s; P_{app} CLPs-AG: $17.2 \pm 1.43 \times 10^{-6}$ cm/s).

The amount of AG that permeated when loaded into liposomes increased about 200 times in respect to the free molecule, as confirmed by the increase of the P_{app} values during the time reported in Figure 5. AG transport across the cell was in a time-dependent manner. The obtained P_{app} data are useful for *in vitro* prediction, as confirmed by the recovery value, which was above 80% in all experiments. The data agrees with PAMPA results.

A combination assay of PAMPA and unidirectional (apical to basal) hCMEC/D3 permeability model can synergistically provide invaluable permeability/absorption assessment of AG. The P_{app} values are greater than those of P_e , due to the presence of an active endocytic mechanism in addition to a passive one, as seen by following uptake studies.

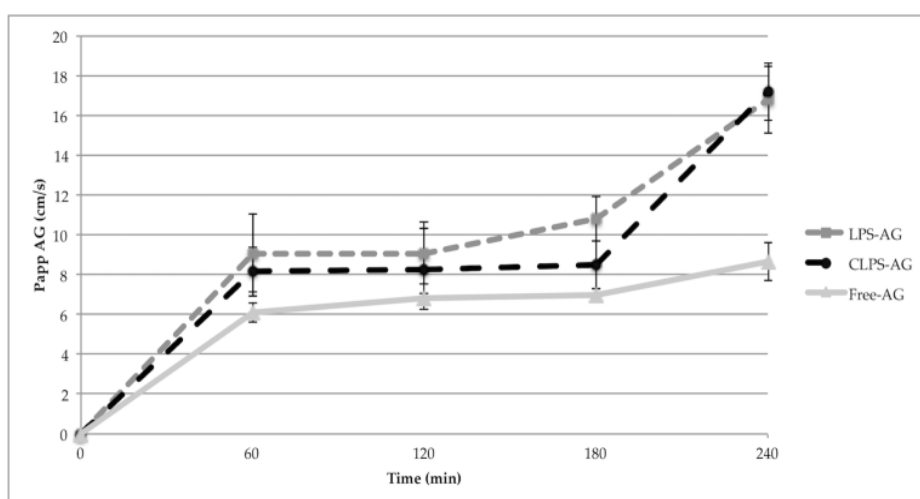


Figure 5. The apparent permeable coefficient of different liposomal formulations for different treatment time in the *in vitro* BBB model.

Data represent means $\pm$ S.D, $n = 3$.

Liposome Uptake by hCMEC/D3 Cells

Figure 6 shows the fluorescence images of hCMEC/D3 cells treated with LPs-6C and CLPs-6C after 2 h of incubation. The images revealed that the probe was internalized and LPs-6C and CLPs-6C were punctually concentrated in intracellular vesicles (endosomes or lysosomes) which can be related to their endocytic mechanism of uptake (Benfer & Kissel, 2012). Besides their cytoplasmic location, green fluorescence indicates that nanoparticles were also transported to the perinuclear area. This result is an important finding for pharmaceutical drug delivery research since the nucleus is the target site for several drugs (Poller et al., 2008).

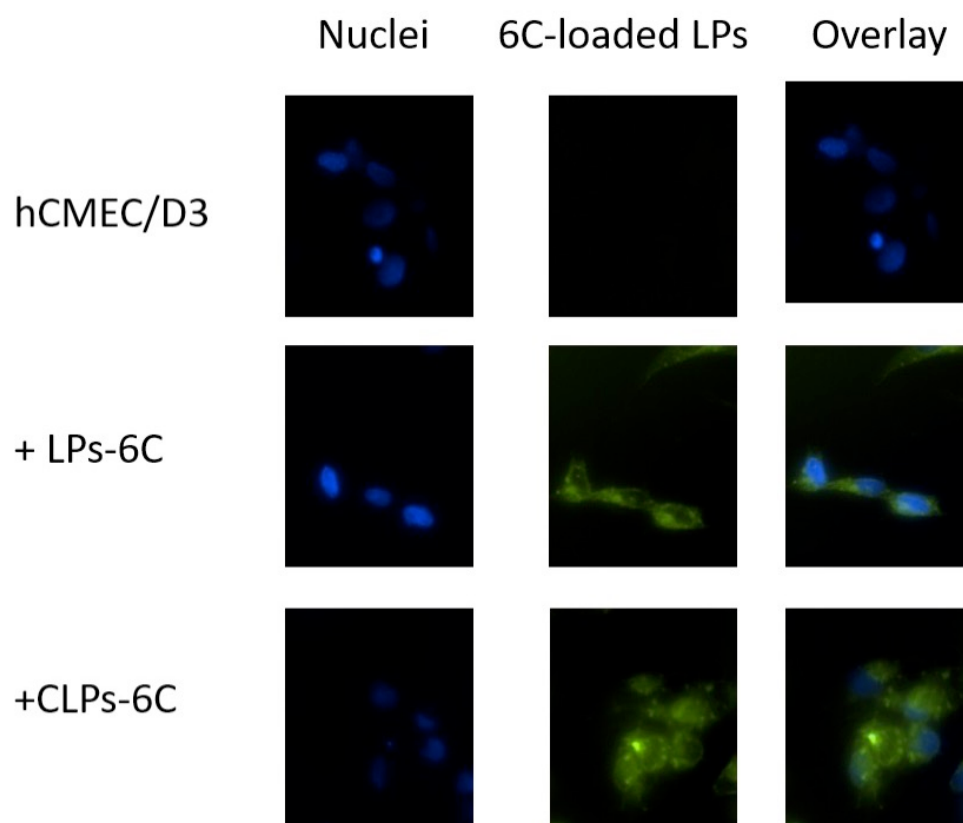


Figure 6. Cellular uptake of LPs-6C and CLPs-6C by hCMEC/D3 cells after 2 h incubation at 37 °C.

Images of nuclei stained with DAPI (blue), 6-Coumarin (green) and their overlay.

Scale bar: 20 μ m.

Concerning the uptake of liposomes by hCMEC/D3 cells, it was different for both liposomes LPs-6C and CLPs-6C: 6.4% and 14.0%, respectively. The result proves that the internalization ability of liposomes increases in the presence of positive charge on the bilayer that improves the binding affinity between the carrier and cellular membrane (Saengkrit et al., 2014).

Furthermore, a group of cells was maintained at 4 °C in the presence of LPs-6C and CLPs-6C, to observe the effect of low temperature, a general metabolic inhibitor. The fluorescence of cells incubated with 6C loaded liposomes in the absence of any inhibitor was considered as 100%, while the fluorescence after incubation in the presence of inhibitors was expressed as a relative percentage compared to the cells without inhibitor. As shown in Figure 7, a significant reduction (about 80%) in hCMEC/D3 cell uptake efficiency was observed at 4 °C for all two formulations as compared with that at physiological temperature, suggesting that their uptake relied on an energy-dependent pathway and it was mediated by endocytosis (Rajendran et al., 2010).

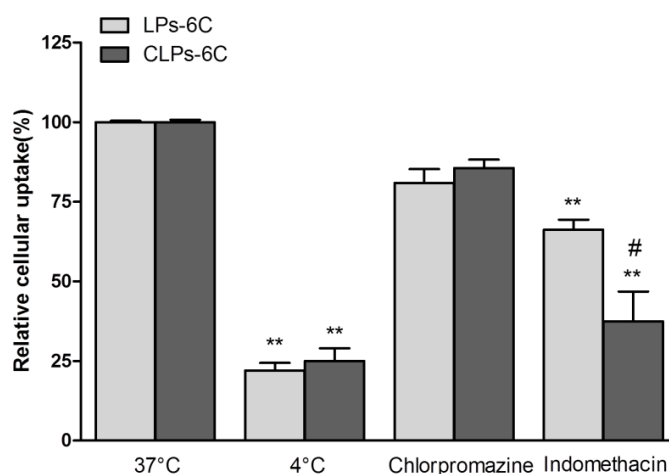


Figure 7. Effect of the temperature (4°C) and different inhibitors on hCMEC/D3 cell internalization pathways of LPs-6C and CLPs-6C after 2 h incubation at 37 °C.

Data represent the mean $\pm$ standard deviation, $n = 3$.

Bars represent the mean $\pm$ SD of at least 6 experiments ** $p < 0.01$ vs. corresponding liposome at 37 °C; # $p < 0.05$ CLPs-6C vs. LPs-6C. (ANOVA + Tukey's test).

To elucidate the endocytic uptake mechanisms, the experiments were also carried out in the presence of endocytic inhibitors, such as chlorpromazine, a clathrin blocker, and indomethacin, a caveolin-dependent endocytosis inhibitor. Liposomes could be internalized into cells by different mechanisms, according to the type of cell, composition, surface charge, and size of the liposome (Huth et al., 2006; Makhlof et al., 2011; K. H. Lin et al., 2016). In our study, the cellular association of the liposomes was significantly influenced by indomethacin, with a reduction in cellular association of about 44% and 63% for LPs-6C and CLPs-6C, respectively (Figure 7). Caveolae-mediated endocytosis is involved in the uptake of liposomes. However, it did not completely inhibit active uptake of the nanoparticles when compared with the results at 4 °C, confirming that cellular uptake of liposomes involved more than on an energy-dependent pathway. In fact, as evidenced in Figure 7, an uptake reduction of 15% for LPs-6C and 20% for CLPs-6C was observed in the presence of chlorpromazine, even if less pronounced than with indomethacin. This result indicates that clathrin-mediated endocytosis is also a mechanism involved in the uptake.

Therefore, the preferential mechanism of the liposomes uptake by hCMEC/D3 cells was found to be caveolae-mediated endocytosis, with a greater effect of inhibitors in the case of CLPs-6C.

CONCLUSIONS

Based on the obtained results concerning size, homogeneity, ζ -potential and EE%, both optimized liposomal formulations of AG are suitable for parenteral administration. The systems showed excellent chemical and physical stability during a month of storage as suspensions or freeze-dried products. The optimized liposomes enhanced solubility and cellular permeability of AG, as demonstrated by *in vitro* tests with PAMPA and hCMEC/D3 cells. Both carriers increase the permeation of AG into the cell without alterations in cell viability and monolayer integrity. The presence of positive charge elevated the cellular internalization of liposomes. Uptake experiments suggest an energy-dependent pathway as a possible transport mechanism across the hCMEC/D3 monolayer, with caveolae-mediated endocytosis, in particular, being the primary mechanism.

hCMEC/D3 cells studies were performed in collaboration with Prof. Domenico E. Pellegrini-Giampietro and Dr. Elisa Landucci (Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).



pharmaceutics



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Stealth and Cationic Nanoliposomes as Drug Delivery Systems to Increase Andrographolide BBB Permeability

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THE EFFECT OF CHITOSAN COATING ON HUMAN SERUM ALBUMIN NANOPARTICLES FOR NOSE-TO-BRAIN DELIVERY

INTRODUCTION

Nowadays the delivery of therapeutics into the brain is a challenging topic. However, most of the drugs in particular hydrophilic molecules and large molecular weight compounds, are not favourably absorbed by the brain due to the presence of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier which determine a selective brain permeability to circulating molecules (Mistry et al., 2009; Y. Chen & Liu, 2012). Among the routes of drug administration, in the last years, the nose-to-brain (NTB) delivery is emerging as a useful and convenient approach that bypasses the BBB and allows the delivery of the drug directly to the brain from the nasal cavity. To confirm this, an increasing number of products, exploiting the nose as the site of administration for the systemic delivery of small and large molecules, are being developed and are reaching the market, such as vaccines, painkillers, anti-migraine and anti-cancer drugs and hormones (Pires et al., 2009; Sonvico et al., 2018).

Nasally administered drugs can rapidly reach the brain through different pathways. The olfactory nerves, which begin at the olfactory epithelium and end at the olfactory bulb, form the major direct pathway for the NTB drug delivery. The trigeminal nerves, which have nerve endings in the respiratory epithelia, form an alternative pathway for the NTB drug delivery. Besides, the substances are also absorbed via the respiratory epithelium into the circulation; if they are capable of crossing BBB, and reach the brain, this represents an indirect pathway for the NTB delivery (Mistry et al., 2009; Bonaccorso et al., 2017; Feng et al., 2018).

However, the NTB delivery is characterized by several limitations such as poor drug permeability across the nasal mucosa, small volume of the nasal cavity, mucociliary clearance, enzymatic degradation, low drug retention time, potential nasomucosal toxicity, the technique of drug administration and deposition and the necessity of a suitable delivery device (Agrawal et al., 2018; Md et al., 2018; Sonvico et al., 2018).

To overcome these drawbacks, pharmaceutical nanotechnologies appear as an ideal formulation strategy for the NTB delivery (Mistry et al., 2009; Agrawal et al., 2018; Feng et al., 2018; Md et al., 2018; Samaridou & Alonso, 2018; Sonvico et al., 2018). In this context, the aim of the present study was the development of human serum albumin nanoparticles (HSA NPs) for the NTB application and the investigation of the effect of chitosan (CS) coating on their performance, in particular with respect to the surface and mucoadhesive properties, stability, drug release modulation, biocompatibility and permeability.

HSA is an endogenous protein, non-toxic, non-immunogenic, biodegradable and biocompatible. HSA can carry poorly water-soluble drugs through aqueous compartments. It displays good mucoadhesive properties, which is crucial for the residence time in the nasal cavity; it exhibits active targeting potential which enhances cellular uptake and it can undergo conformational changes to escape from low and high pH values and enzymatic degradation (Elzoghby et al., 2012; Bergonzi et al., 2016; Tan & Ho, 2018). Furthermore, as reported in literature, albumin is an ideal material for the development of nanoparticles to enhance the pharmacological effects of plant-derived molecules, such as, curcumin (Jithan et al., 2011; Camargo et al., 2018), apigenin (Papay et al., 2017), andrographolide (Guccione et al., 2017), chrysin (Ferrado et al., 2018) and berberine (Elgohary et al., 2018). Most of these molecules exhibit promising neurological activities, but their clinical application is limited due to low bioavailability and high instability. To the best of our knowledge, HSA NPs were studied for the NTB delivery of the anti-Alzheimer drugs tacrine and *R*-flurbiprofen (Luppi et al., 2011; Wong & Ho,

2018). However, CS-coated HSA NPs (CS-HSA NPs) for NTB application have not been studied yet. Proteins and polysaccharides hold great potential for the development of nanocarriers for drug delivery purposes based on their unique biocompatibility, biodegradability, ease of functionalization, improved biodistribution and minimal toxicity profiles (Esfandyari-Manesh et al., 2016; Li et al., 2018; Gaber et al., 2018). In particular, CS, a polysaccharide derived from deacetylation of chitin, was employed because it has the additional effect of reversibly opening tight junctions, with the potential to increase the drug permeation across the nasal mucosa and the extracellular transport along the olfactory and trigeminal nerves. Besides, CS is positively charged at nasal slightly acid pH value and it forms electrostatic interactions with the negatively charged epithelial cells to reduce the clearance from the nasal epithelium. Furthermore, CS absorbs water from the mucus layer in the nasal cavity, swells and forms a gel-like layer prolonging drug residence time at the site of absorption and improving its bioavailability (Casettari & Illum, 2014; Rassu et al., 2015; Esfandyari-Manesh et al., 2016; Sonvico et al., 2018)

The developed formulations were physically and chemically characterized by Dynamic Light Scattering (DLS), Electrophoretic Light Scattering (ELS), Transmission Electron Microscope (TEM) and High Performance Liquid Chromatography (HPLC) analyses. The freeze-drying process was optimized and stability studies upon storage were performed. The mucoadhesion properties were assessed through the turbidimetric method and the evaluation of the ζ -potential. HSA NPs and CS-HSA NPs were loaded with sulforhodamine B sodium salt as a model of a hydrophilic drug and the effect of CS coating was investigated performing release studies, permeation and uptake experiments using Caco-2 and hCMEC/D3 cells as model of the nasal epithelium and BBB, respectively (Eigenmann et al., 2013; Rassu et al., 2015; Rassu et al., 2017; Guccione et al., 2017). Furthermore, *ex vivo* diffusion experiments were performed using rabbit nasal mucosa in a Franz-type permeation apparatus.

MATERIALS

Human Serum Albumin (HSA), Sulforhodamine B sodium salt (Sulfb), Chitosan Low Molecular Weight (CS LMW), Sodium chloride (NaCl), Sodium Hydroxide (NaOH), Phosphate buffered saline (PBS) bioperformance certified, Mucin from porcine stomach, Sodium azide, Chlorpromazine, Indomethacin, D-Mannitol, D(+)-Glucose anhydrous, D(+)-Sucrose, Acetonitrile HPLC grade, Methanol HPLC grade, Formic acid analytical grade, Hydrochloric acid (HCl) analytical grade, Ethanol analytical grade, Glutaraldehyde, Glacial Acetic Acid, were purchased from Sigma-Aldrich (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, CT, USA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, PA, USA). Dialysis kit was from Spectrum Laboratories, Inc. (Breda, The Netherlands).

METHODS

Preparation of human serum albumin nanoparticles

HSA NPs were prepared by the well-established desolvation-coacervation technique (Weber et al., 2000; Langer et al., 2003) with some modifications.

30 mg of HSA were dissolved in 3 mL of 10 mM NaCl solution under magnetic stirring at a speed of 500 rpm. The pH value was adjusted between 8 and 9 with NaOH 0.1 N. Then, ethanol was added at a constant rate using a syringe until turbidity appeared in the solution. The obtained coacervates were hardened by the addition of 5% glutaraldehyde solution, corresponding to 100% of the amount required for the cross-linking of the 60 amino groups of HSA. After 2 h under magnetic stirring at room temperature, ethanol was vacuum evaporated and the pellet was redispersed in distilled water.

In addition, fluorescent NPs were prepared, adding sulforhodamine B sodium salt ($\lambda_{\text{ex}}=565$ nm, $\lambda_{\text{em}}=586$ nm in water, SulfB) to the HSA solution. The resulted mixture was incubated for 2 h under magnetic stirring at 500 rpm, before the addition of ethanol (Bergonzi et al., 2016).

Preparation of chitosan-coated nanoparticles

CS-HSA NPs were obtained exploiting the ionic interactions between negatively charged HSA NPs and positively charged CS (Esfandyari-Manesh et al., 2016).

Firstly, CS was dissolved in 1% v/v glacial acetic acid at the concentration of 1 mg/mL. After 24 h under stirring at room temperature, the solution was filtered through a 0.45 μm membrane and then, the pH was adjusted to 4.5 with NaOH diluted solution.

Afterwards, 5 mL of CS solution was added to an equal volume of HSA NPs suspension under magnetic stirring at a speed of 250 at room temperature. The stirring was maintained for 15 min to allow the stabilization of the CS coating.

Particle Size, Polydispersity Index and ζ -Potential

The evaluation of the particle size and the polydispersity index (Pdl) was performed by dynamic light scattering using a Zsizer Nanoseries ZS90 (Malvern Instrument, Worcestershire, UK) at a fixed angle of 90° and at 25 °C. The ζ -potential, indicating the electric charge on the NPs surface and the physical stability of the formulations, was calculated by the Helmholtz–Smoluchowski's equation using the same instrument. Before the measurements, the samples were diluted with distilled water. The analyses were performed in triplicate.

Transmission Electron Microscopy

The shape and the surface structure of HSA NPs and CS-HSA NPs were investigated by transmission electron microscopy (TEM, Jeol 1010, Tokyo, Japan). The samples were properly diluted with distilled water, then they were dropped on a 200 mesh carbon film-covered copper grid and negatively stained with a phosphotungstic acid aqueous solution (1% w/v). After drying, the grid containing the samples were observed with the TEM.

Determination of nanoparticle yield

In order to evaluate the amount of HSA transformed into nanoparticles, 1.5 mL of HSA NPs and CS-HSA NPs were centrifuged at 18000 rpm for 45 min. Then, the obtained supernatants were analyzed by HPLC (Merodio et al., 2001).

HSA analyses were performed using an HP 1200 liquid chromatograph equipped with a diode-array-detector (DAD) and controlled with an HP 9000 workstation (Agilent Technologies, Santa Clara, CA, USA). A Luna Omega Polar (150 mm × 3 mm, 5 µm) (Agilent Technologies, Santa Clara, CA, USA) analytical column was used. The mobile phases were: (A) formic acid/water pH 3.2 and (B) acetonitrile with 0.5 mL/min flow rate. The multi-step linear solvent gradient applied was: 0-2 min, 90% A, 10% B; 2-7 min, 90-60% A, 10-40% B; 7-17 min, 60-30% A, 40-70% B; 17-20 min, 30-10% A, 70-90% B; 20-22 min 10% A, 90% B; 22-27 min, 10-90% A, 90-10% B with post-time of 8 min.

The chromatograms were acquired at a wavelength of 280 nm (Merodio et al., 2001). The linearity range of responses of HSA dissolved in distilled water was determined on five concentration levels (from 0.1 to 1 mg/mL) with three injections for each level. The curve showed a coefficient of linear correlation above than 0.999.

Determination of Encapsulation Efficiency

The encapsulation efficiency (EE%) was determined by the dialysis bag method (Righeschi et al., 2014). The membrane (cut-off 3.5 kD), filled with 2 mL of the samples, was kept for 1 h in 1 L of distilled water, to remove the free SulfB. Then the samples were opportunely diluted with acetonitrile/water 70:30 mixture, sonicated in an ultrasonic bath for 15 min and analyzed by HPLC.

SulfB analyses were performed using the same instrument, column and analytical method reported for HSA analysis, but the chromatograms were acquired at 560 nm.

The encapsulation efficiency was calculated by the following equation:

$$\text{Encapsulation efficiency \%} = \frac{\text{SulfB encapsulated}}{\text{Total SulfB}} \times 100$$

Freeze-drying process

One aliquot of HSA NPs and CS-HSA NPs was frozen with liquid nitrogen and then moved to the freeze-drier. Others three aliquots were freeze-dried after the addition of D-Glucose anhydrous (2% w/v) or D-Mannitol (2% w/v) or D(+)-Sucrose (% w/v) as cryoprotective agents (Anhorn et al., 2008). The drying time was 24 h.

After the freeze-drying process, each sample was reconstituted with the original volume of distilled water, vortexed for few minutes to ensure the complete redispersion of the powder (Anhorn et al., 2008) and analyzed for particle size, polydispersity and ζ -potential.

Storage stability studies

Stability studies were conducted over a time period of three months. Particle size, PDI and ζ -potential of HSA NPs and CS-HSA NPs as suspension, at 4°C, and as freeze-dried products, stored at room temperature, were evaluated.

Phase separation and turbidity were also considered in the case of the suspensions, while the freeze-dried samples were evaluated for the appearance of the powder and for the redispersibility time.

Mucoadhesion properties

To evaluate and compare the mucoadhesion properties of HSA NPs and CS-HSA NPs in the presence of mucin, the turbidimetric method (Bonferoni et al., 2010) and the evaluation of the ζ -potential (indirect method) were applied (Vieira et al., 2018).

An equal volume of nanoparticles suspension and 1% w/v mucin aqueous solution were mixed. Then, the resulted samples were vortexed for 1 min and incubated in a water bath for 1 h at 37°C. The turbidity of the mixtures was spectrophotometrically evaluated at $\lambda = 500$ nm. The absorbencies of individual mucin and nanoparticles suspensions were also recorded. The interaction between mucin and HSA NPs or CS-HSA NPs was calculated using the following equation:

$$\Delta A = A - A_{\text{theor}}$$

where ΔA is the absorbance difference, A is the effective absorbance of HSA NPs-mucin and CS-HSA NPs-mucin mixtures and A_{theor} represents the sum between the individual absorbance of mucin and nanoparticles suspensions. If $\Delta A = 0$, no interactions occur, whereas if $\Delta A \gg 0$ there is a strong interaction between nanoparticles and mucin.

The ζ -potential was measured as previously reported.

In vitro release studies

In vitro release studies were performed in physiological pH conditions (PBS pH 7.4) using the dialysis bag method. For these studies regenerated cellulose membranes with a molecular cut-off of 3.5 kD were chosen for retaining the nanoparticles and allowing for the diffusion of the entrapped compound into the dissolution medium. HSA-NPs, CS-HSA NPs or SulfB aqueous solution (2 mL) were added into dialysis membranes and placed in 200 mL of release medium. Thus, the systems were incubated at 37°C with stirring at 150 rpm. At predetermined time intervals (0, 0.5, 1, 2, 4, 5, 8 and 24 h) 1 mL of the medium was withdrawn and replaced with the same volume of fresh release medium maintained at 37°C to preserve sink conditions. SulfB released at each time interval was quantified using HPLC. All experiments were performed in triplicate.

In order to understand the kinetic and the mechanism of drug release from HSA NPs and CS-HSA NPs, Hixson-Crowell model, Higuchi model, first- and zero-order mathematical models were used and the best-fitted model was selected based on the higher regression coefficient (R^2) value for the release data.

Caco-2 experiments

Caco-2 cell line

The colorectal adenocarcinoma cell line Caco-2 was purchased from American Tissue Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Rodano, Milan, Italy) with 20% fetal bovine serum (FBS) (Thermo Fisher Scientific, Rodano, Milan, Italy), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Rodano, Milan, Italy) in 5% CO₂ at 37 °C.

Cell Culture Experiments

An MTS assay was performed to determine the cell viability after exposure to HSA NPs and CS-HSA NPs at different dilutions (from 1:10 to 1:40) for 2 and 24 h, as previously described (Bigagli et al., 2017). The relative cell viability was expressed as a percentage of the untreated control group.

Transport Studies

For the permeation studies, Caco-2 cells were seeded into 12-well PET transwell plates (1.13 cm² growth surface area and pore size 0.4 µm, Greiner Bio-One, Milan, Italy) at a density of 2×10^4 cells/cm² and grown for 21 days to form a confluent monolayer.

SulfB-HSA NPs and SulfB-CS-HSA NPs diluted 10 times were incubated for 4 h in the apical compartment. The integrity of the cellular barrier was assessed using Lucifer Yellow (LY) permeability test (Iacomino et al., 2013).

Samples of media were collected from the basal compartment of each transwell plate and replaced with fresh HBSS at 0 (before sample addition), 60, 120, 180 and 240 min. The SulfB concentration in these samples was determined by HPLC and adjusted to reflect the change in volume at each successive sampling point. LY measurements were also taken at the end of the experiment.

Uptake Studies

To identify the uptake mechanism of the developed nanoparticles, Caco-2 cells were pre-incubated with sodium azide (an ATP synthesis inhibitor, 1 μ M), chlorpromazine (a clathrin blocker, 15 μ M), and indomethacin (a caveolin-dependent endocytosis inhibitor, 25 μ M) for 30 min followed by the addition of SulfB-HSA NPs or SulfB-CS-HSA NPs 1:10 for 1 h, or maintained at 4°C during the NPs exposure. At the end of the treatments, the amount of SulfB was measured on cellular lysate by HPLC analyses.

hCMEC/D3 experiments

hCMEC/D3 cell line

This cell line (Millipore Cat. # SCC066) derives from human temporal lobe microvessels isolated from tissue excised during surgery for epilepsy control. Cells were seeded in a concentration of 2.5×10^4 cells/cm² and grown at 37 °C in an atmosphere of 5% CO₂ in 25 cm² rat tail collagen type I coated culture flasks. EndoGRO TM- MV Complete Media Kit (Cat. # SCME004) supplemented with 1 ng/mL FGF-2 (Cat. #GF003) was changed every three days and cells were grown until they reached 90 % confluency. Cells were passaged at least twice before use. Confluent hCMEC/D3 cells were split by Accumax TM Cell Counting Solution in DPBS.

MTT assay

To assess cell viability after HSA NPs and CS-HSA NPs exposure, an MTT assay was performed (Conti et al., 2010; Landucci et al., 2016). Cells were seeded in a 24-well plate (6×10^4 cells /cm²) pre-coated with Collagen Type I, Rat Tail (Cat. #08-115) and grown at 37°C in an atmosphere of 5% CO₂ in EndoGRO™ Basal Medium (EBM-2). When the cells were approximately 70-80% confluent, they were incubated with different concentrations of HSA NPs and CS-HSA NPs, obtained by dilution (1:10, 1:100 and 1:500) of the formulation in EBM-2 for 2 and 24 h. The NPs formulations were previously filtered through 0.4 µm sterile filter units. The medium of each well was separated from the cells and stored for LDH assay, and cells were treated with 1 mg/mL of MTT for 1 h at 37 °C and 5% CO₂. Finally, DMSO was added to dissolve MTT formation and absorbance was measured at 550 and 690 nm. Cell viability was expressed as a percentage compared to the cells incubated only with EBM-2 (positive control). Triton X-100 was used in the MTT assay as the negative control since its detergent action disrupts the cells.

LDH assay

Cytotoxicity after HSA NPs and CS-HSA NPs exposure was verified with LDH assay. The medium resulting from incubation of NPs with cells was centrifuged (250 g, 10 min at RT) and the supernatant separated from the deposited cells in each well. This centrifugation process allowed us to remove any wastes and cellular debris as well as HSA NPs and CS-HSA NPs. The release of LDH into culture supernatants was detected by adding catalyst and dye solutions of a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, USA). The absorbance values were recorded at 490 nm and 690 nm. Cytotoxicity was expressed as a percentage compared to the maximum LDH release in the presence of triton X-100 (positive control). EBM-2 was used as the negative control since no cytotoxicity was detected in such conditions.

hCMEC/D3 cell culture for transwell permeability studies

High-density pore (2×10^6 pores/cm²) transparent PET membrane filter inserts (0.4 μ m, 23,1 mm diameter, Falcon, Corning BV, Netherlands) were used in 6-well cell culture plates (Falcon, Corning, Amsterdam, Netherlands) for all transcytosis assays. The transparent PET membrane filter inserts were coated with rat tail collagen type I at a concentration of 0.1 mg/mL and incubated at 37°C for 1 h prior to cell barrier coating. Inserts were subsequently washed with PBS and incubated for 1 hour, after which PBS was removed and replaced with the assay medium. The inserts were calibrated for at least 1 hour with assay medium at 37 °C. Optimum media volumes were calculated to be 1 mL and 1.2 mL respectively for the apical and basolateral chambers. The transwell inserts were calibrated with assay medium for 1 h, then the medium was removed and hCMEC/D3 cells were seeded onto the apical side of the inserts at a density of 6×10^4 cells /cm² in 1 mL assay media. 1.2 mL of fresh medium was added to the basolateral chamber. hCMEC/D3 monolayers were used as a permeability assay for SulfB-HSA NPs and SulfB-CS-HSA NPs. Fluorescein sodium salt (NaF) was considered at a concentration of 10 μ g/mL as integrity control marker with a known permeability coefficient (P_{app}) for this cell line (Guccione et al., 2017). The integrity of monolayer cells was also confirmed by observation of cultures under phase-contrast microscopy or bright-field optics using of transparent membranes. The image was observed using an inverted microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) with a low-power objective (20X). The images were digitized using a video image obtained with a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA).

For permeability studies, SulfB-HSA NPs and SulfB-CS-HSA NPs, diluted 10 times were tested and incubated for 1, 2 and 3 h in the apical donor compartment. At the end of the incubation, the amount of NaF and SulfB were quantified both in apical and basolateral compartments by HPLC-FLD or HPLC-DAD method. NaF was analysed using the same apparatus and the analytical method described in the previous chapter.

Cellular uptake of SulfB-HSA NPs and SulfB-CS-HSA NPs

To identify the uptake mechanism of the developed nanoparticles, hCMEC/D3 cells were pre-incubated with sodium azide (an ATP synthesis inhibitor, 1 μ M), chlorpromazine (a clathrin blocker, 15 μ M), and indomethacin (a caveolin-dependent endocytosis inhibitor, 25 μ M) for 30 min followed by the addition of SulfB-HSA NPs or SulfB-CS-HSA NPs 1:10 for 2h, or maintained at 4°C during the NPs exposure. At the end of the treatments, the amount of SulfB was measured on cellular lysate by HPLC analyses.

Ex vivo transport experiments across rabbit nasal mucosa

Ex vivo transport experiments were performed using rabbit nasal mucosa in a Franz-type diffusion apparatus (0.58 cm² permeation area, VETROTECNICA S.r.l., Padova, Italy) (Russo et al., 2006; Bortolotti et al., 2009; Giuliani et al., 2018). On the day of the experiment, rabbit heads were collected from a local slaughterhouse (Pola S.r.l, Finale Emilia, Italy) and transported to the laboratory in a refrigerated box. The extraction procedure, completed within 2 h from the animal's death, is described elsewhere (Balducci et al., 2013). The nasal mucosa was rinsed with PBS pH 7.4 and immediately inserted between the donor and the receptor compartments of the Franz cell, with the mucosal side facing the donor.

In order to assess the proper cell assembly as well as the mucosa integrity, the donor compartment was filled with saline solution, checking that no liquid passed to the empty receptor due to improper mounting or lack of tissue integrity. Afterwards, the receptor compartment was filled with PBS pH 7.4 and the assembled system was allowed to equilibrate at 37°C for half an hour before introducing the formulation in the donor compartment (SulfB-HSA NPs, SulfB-CS-HSA NPs and SulfB aqueous solution).

The experiments were carried out over a 4 h and 24 h period of time. This experiment duration was consistent with the data in literature about tissue viability and drug transport experiments across animal excised nasal mucosa, employing nano-drug delivery systems (Bechgaard et al., 1992; Schmidt et al., 2000; Ved & Kim, 2011, Bari et al., 2015; Javia & Thakkar, 2017; Raj et al., 2018).

At predetermined time points, a fixed volume of receptor solution (0.5 mL) was withdrawn, and the receptor compartment refilled with an equivalent volume of fresh PBS. At the end of the experiment, aiming to calculate the mass balance (sum of the amounts of SulfB recovered from receptor, donor and membrane), the residual formulation on the membrane was quantitatively recovered by rinsing the donor compartment with PBS. In addition, SulfB retained at the membrane nasal mucosa was extracted with distilled water using an IKA T25 digital Ultra-Turrax (IKA-Works, Staufen im Breisgau, Germany) for 10 min and then an ultrasound bath for 30 min. All the samples were analyzed by HPLC.

The permeability coefficient (P_e) of SulfB across the mucosa in steady-state conditions was calculated according to the solution of Fick Equation (Bortolotti et al., 2009):

Statistical analysis

The experiments were repeated three times and the results expressed as a mean $\pm$ standard deviation. Statistical significance of Caco-2 and hCMEC/D3 cell viability, permeability studies and cellular uptake was analyzed using one-way ANOVA followed by the post hoc Tukey's w-test for multiple comparisons. All statistical calculations were performed using GRAPH-PAD PRISM v. 5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (P) of < 0.05 was considered significant.

RESULTS AND DISCUSSION

HSA NPs characterization

HSA NPs were obtained by the most widely used desolvation-coacervation technique (Weber et al., 2000; Langer et al., 2003).

HSA was dissolved in a 10 mM NaCl solution and the pH was adjusted between 8 and 9 considering that the particle size decreased with increasing pH of the HSA solution (Langer et al., 2003). The hydrodynamic diameter is one of the most critical parameters for the development of suitable nanocarriers for nose to brain delivery. Indeed, only small particles can be transported to the brain via the olfactory or the trigeminal nerves (Mistry et al., 2009). NaCl solution was applied due to the difficulty to obtain the correct pH value in the absence of salts (Westcott, 1978), while other buffers interfere with the desolvation and cross-linking process (Langer et al., 2003).

Ethanol was selected as the desolvating agent to achieve albumin desolvation. The dielectric constant, dipole moment and solubility parameter make ethanol an ideal solvent to enter into the hydrophobic region of HSA, by disrupting the hydrophilic layer of the protein in water, and to induce the denaturation of HSA and consequently the formation of NPs (Mohammad-Beigi et al., 2016). Besides, ethanol was selected considering its less toxicity respect to other organic solvents proposed as desolvating agents, i.e., acetone, methanol and acetonitrile (Likhodii et al., 2003; Adriaens et al., 2005).

Glutaraldehyde aqueous solution was used as cross-linker agent (Langer et al., 2003). Among the different concentrations were tested (from 50% to 200% of the calculated amount required for the cross-linking of the amino groups of HSA), the best results concerning particle size, homogeneity, yield and reproducibility were obtained with a concentration of 100% (Table 1).

TEM observations confirmed the presence of spherical HSA NPs with uniform size distribution in the range of 230-250 nm, according to DLS measurements (Figure 1).

The surface charge of the developed HSA NPs was negative due to the presence of the terminal carboxyl groups of the protein. The high ζ -potential value indicates the presence of strong repulsive forces which can prevent the aggregation of the NPs, increasing the stability of the formulation (Table 1). Besides, the negative surface charge is crucial for further surface modification step.

Sample	Size (nm)	PdI	ζ -potential (mV)	EE%	Yield %
HSA NPs	241 $\pm$ 19	0.06 $\pm$ 0.04	-47 $\pm$ 3	-	93 $\pm$ 1
SulfB-HSA NPs	244 $\pm$ 12	0.11 $\pm$ 0.02	-44 $\pm$ 5	65 $\pm$ 2	95 $\pm$ 2

Table 1. Physical and chemical characterization of empty and SulfB-loaded HSA NPs.
Data displayed as mean $\pm$ SD; n=3.

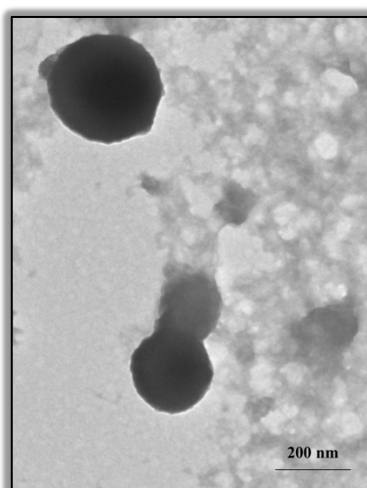


Figure 1. TEM image of HSA NPs.
Scale 200 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

The nanoparticle yield, determined by HPLC analyses (Merodio et al., 2001), resulted very high, indicating that almost all HSA was converted into NPs. Thus, the obtained result demonstrated the success of the preparative process.

SulfB, a lipophilic fluorescent dye, was incorporated into HSA NPs as hydrophilic drug model to perform release studies and *in vitro* experiments with Caco-2 and hCMEC/D3 cells and to investigate the permeation across the rabbit nasal mucosa. SulfB was selected because it does not exhibit pH-dependent absorption or fluorescence over the range of 3 to 10 and it is highly soluble in the media employed in the *in vitro/ex vivo* assays.

As reported in Table 1, the addition of SulfB (final concentration 0.1 mg/mL) did not cause a worsening on size and homogeneity of HSA NPs.

CS-HSA NPs characterization

CS-coated HSA NPs were obtained by the electrostatic interaction between negatively charged HSA NPs and positively charged CS (Esfandyari-Manesh et al., 2016) . Low molecular weight CS was used according to Bonaccorso et al., 2017 which tested *in vivo* CS-coated NPs, using the same kind of polymer.

The increase in particle size and the inversion of ζ -potential value clearly indicated the interaction between HSA NPs and CS (Table 2). The high ζ -potential, either positive or negative, is related to a stable formulation in which the electrostatic repulsions between NPs with the same electrical charge prevent their aggregation. The positive surface charge of CS-HSA NPs also makes them able to further interact with negatively charged cell membranes and to improve their mucoadhesive properties.

Different CS concentrations were tested (from 1 mg/mL to 3 mg/mL) and the increase in particle size (above than 300 nm) and a loss of homogeneity (PdI > 0.3) were observed increasing the CS concentration, while the ζ -potential resulted almost unchanged. Thus, CS was employed at the concentration of 0.1 mg/mL (Table 2).

TEM micrographs provided information on morphology and dimensions of the NPs and showed the presence of CS on the surface of HSA NPs as a grey shell around them (Fonte et al., 2011; Bugnicourt & Ladaviere, 2017) (Figure 2).

Other physicochemical parameters of HSA NPs such as PDI, EE% and the nanoparticle yield were not affected but the presence of CS.

Sample	Size (nm)	PdI	ζ -Potential (mV)	EE%	Yield %
CS-HSA NPs	261 ± 8	0.10 ± 0.07	$+45 \pm 1$	-	94 ± 1
SulfB-CS-HSA NPs	267 ± 6	0.12 ± 0.02	$+44 \pm 1$	63 ± 1	95 ± 3

Table 2. Physical and chemical characterization of empty and SulfB-loaded CS-HSA NPs.
Data displayed as mean $\pm$ SD; n=3.

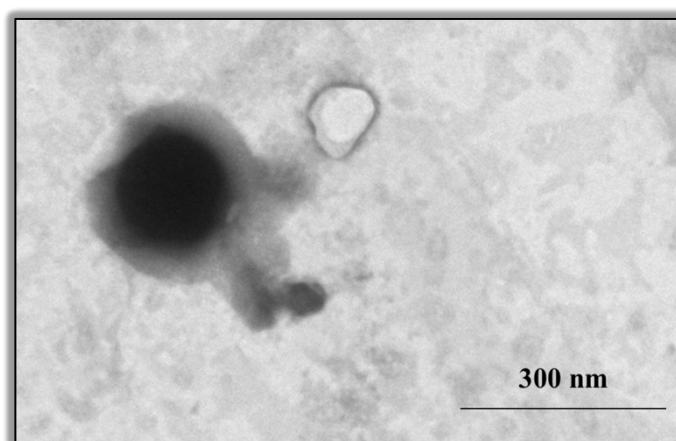


Figure 2. TEM image of CS-HSA NPs.
Scale 300 nm.

TEM analyses were performed thanks to the collaboration with Dr. Maria Cristina Salvatici, Electron Microscopy Centre (Ce.M.E.), ICCOM, CNR, Sesto Fiorentino, Florence, Italy.

Freeze-drying process

Freeze-drying is a suitable technique to improve the long-term storage stability of nanoparticles and to reduce the possible microbial contamination due to the presence of the aqueous medium. In this work, HSA NPs and CS-HSA NPs were freeze-dried without cryoprotectants and in the presence of sugars or sugar alcohols such as glucose, sucrose and mannitol. Before the freeze-drying process, the samples were frozen with liquid nitrogen and then moved to the freeze-drier. Fast freezing rate was preferred to slow freezing rate (such as the freezer) because it avoids the formation of large ice crystals which damage the NPs and reduces the contact time between the NPs preventing their aggregation and resulting in smaller particle size (Abdelwahed et al., 2006; Lee et al., 2009).

In the absence of cryoprotectant, a significant increase in size and a loss of homogeneity was observed for both formulations (Table 3). This result confirmed the advantage of cryoprotectants in preventing the aggregation of albumin NPs during the freeze-drying process (Anhorn et al., 2008).

Freeze-dried product	Size (nm)	PdI	ζ-potential (mV)
HSA NPs	> 500 nm	> 0.5	-
HSA NPs + Glucose	269 ± 3	0.05 ± 0.01	-45 ± 2
HSA NPs + Mannitol	262 ± 5	0.06 ± 0.01	-44 ± 1
HSA NPs + Sucrose	261 ± 1	0.06 ± 0.01	-45 ± 1
CS-HSA NPs	> 500 nm	> 0.5	-
CS-HSA NPs + Glucose	276 ± 5	0.12 ± 0.02	+31 ± 1
CS-HSA NPs + Mannitol	285 ± 2	0.11 ± 0.01	+17 ± 3
CS-HSA NPs + Sucrose	269 ± 3	0.06 ± 0.01	+35 ± 1

Table 3. Physical parameters of HSA NPs and CS-HSA NPs after the freeze-drying process without and with different cryoprotectants.

Data displayed as mean ± SD; n=3.

All the cryoprotectants were tested at the concentration of 2% w/v and among them, the best results concerning particle size, homogeneity and ζ -potential were obtained in the presence of sucrose (Table 3). This was an interesting finding considering that for protein-based formulations, non-reducing compounds such as mannitol, and sucrose are generally preferred to avoid potential Maillard reaction of the cryoprotectant with the protein (Costantino, 2004).

Sucrose resulted better than mannitol in particular for CS-HSA NPs, so it was selected for storage stability studies. A possible reason for this result could be the tendency of mannitol to crystallize during the freeze-drying process (Costantino, 2004).

To be precise, a slight increase in size was also found in the presence of sucrose; however, HSA NPs and CS-HSA NPs maintained physical characteristics suitable for the intranasal application.

Storage stability studies

Physical stability of HSA NPs and CS-HSA NPs suspensions and as freeze-dried product was monitored during three months.

After three months at 4°C both HSA NPs and CS-HSA NPs suspensions were stable. The final values of particle size and PdI were 238 ± 5 and 0.10 ± 0.01 , respectively, for HSA NPs, while for CS-HSA NPs were 265 ± 2 and 0.11 ± 0.01 , respectively. No significant changes were also found regarding ζ -potential after 3 months of storage: -47 ± 1 for HSA NPs and $+43 \pm 2$ for CS-HSA NPs.

A similar study was performed on freeze-dried NPs. Both products maintained almost the same initial physical characteristics: size 260 ± 3 nm, PdI 0.07 ± 0.01 and ζ -potential of -40 ± 1 mV, in the case of HSA NPs; and size 265 ± 1 nm, PdI 0.06 ± 0.01 and ζ -potential of $+37 \pm 2$ mV, in the case of CS-HSA NPs.

These results demonstrate that the developed NPs can be stored as suspension in the fridge and potentially even at room temperature as freeze-dried products for long periods.

Mucoadhesion studies

Turbidity analyses and the evaluation of the ζ -potential were synergistically applied to investigate and compare the mucoadhesion properties of CS-HSA NPs and uncoated NPs.

As clearly reported in Figure 3, a significant increase in absorbance difference (ΔA) was found in the case of CS-HSA NPs indicating a strong interaction between CS and mucin. This is due to the formation of ionic bonds between the positively charged amino groups present in CS and the negatively charged carboxyl and sulphate groups of the mucin (Luo et al., 2015; Vieira et al., 2018).

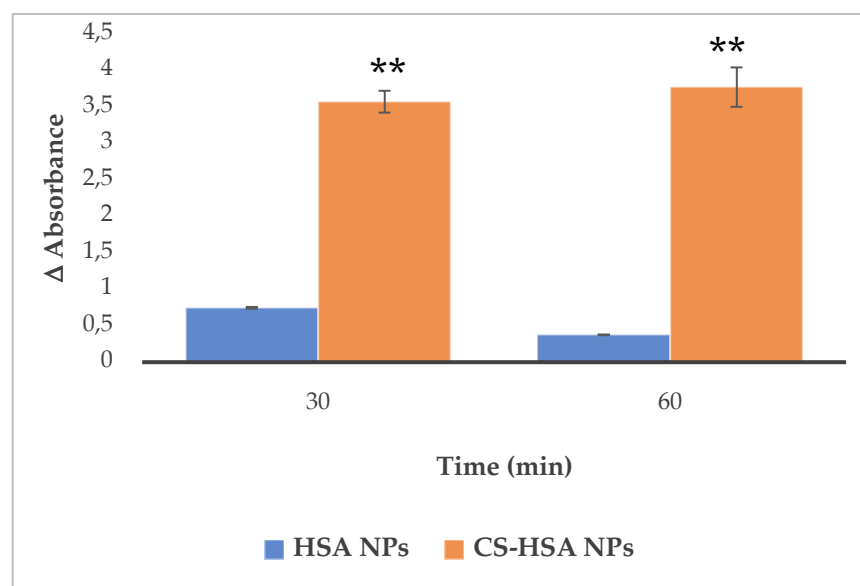


Figure 3. Turbidimetric method.

Absorbance difference of mucin admixed with HSA NPs and CS-HSA NPs.

Data displayed as mean $\pm$ SD; n=3. ** $p < 0.01$ vs. HSA NPs (ANOVA + Tukey's test).

ζ -potential analyses confirmed the data obtained by the turbidimetric method. As evidenced in Figure 4, positive charges of CS-HSA NPs were neutralized by the negative charge of mucin. On the other hand, uncoated NPs remain negatively charged once the interaction with mucin occurs at lower extension (Grenha et al., 2005; Vieira et al., 2018).

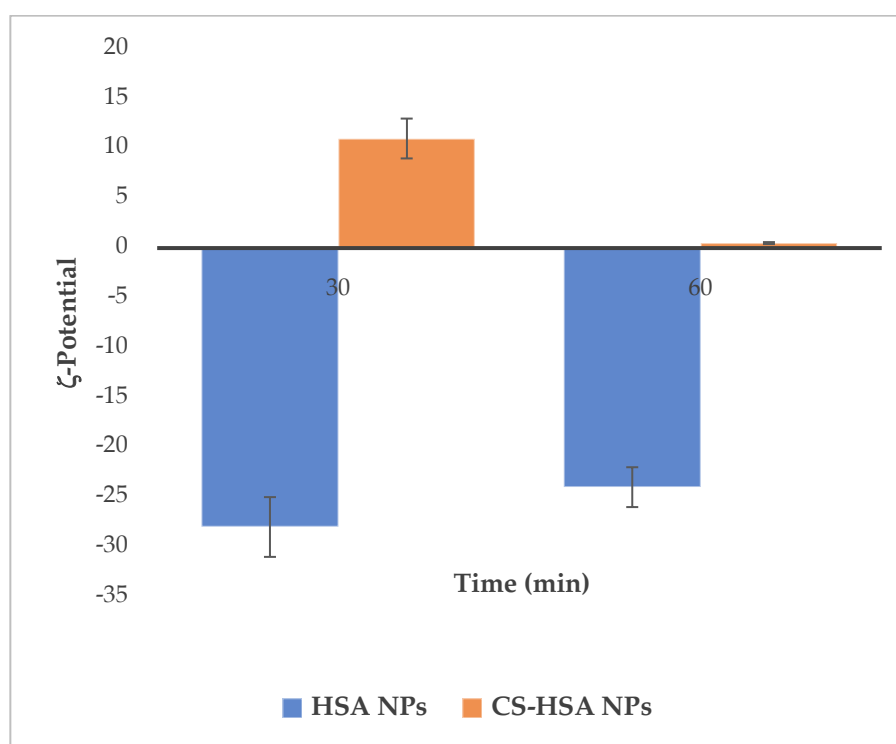


Figure 4. Indirect method.

ζ -potential of HSA NPs and CS-HSA NPs after incubation with mucin.

Data displayed as mean $\pm$ SD; n=3.

***In vitro* release experiments**

In vitro release profiles of SulfB aqueous solution, from HSA NPs and from CS-HSA NPs through regenerated cellulose membranes are reported in Figure 5. In the case of the solution, 100% of SulfB was released after 2 h, while in the case of both nanoparticle formulations the immediate release of the entrapped molecule (known as burst effect) was very low. This represents an important finding because it suggests that the drug leakage during *in vivo* delivery would be minimal. Looking more closely, the release of SulfB from CS-HSA NPs started quite slower respect to HSA NPs, then, after about four hours, the release profiles of the formulations became similar as a consequence of CS hydration and swelling (Figure 5) (Singh & Lillard, 2009; Raj et al., 2018).

As previously reported, the release kinetics of SulfB (employed as a model of a hydrophilic drug) were investigated by Hixson-Crowell, Higuchi, first- and zero-order mathematical models. The results revealed that the release of SulfB from both formulations was diffusion-controlled, as indicated by higher R-squared (R^2) values in the Higuchi model (0.839 for HSA NPs and 0.884 for CS-HSA NPs).

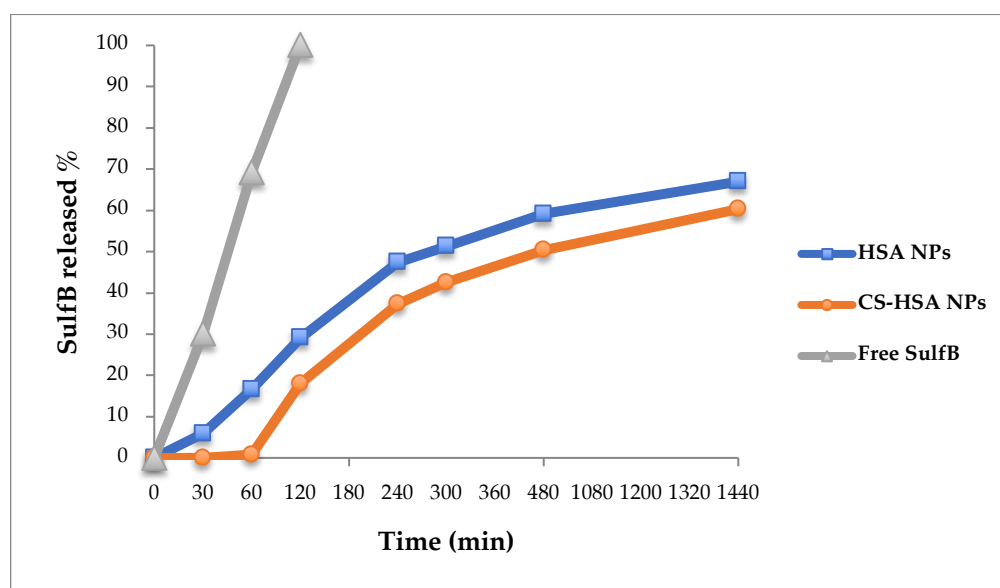


Figure 5. *In vitro* release profiles of SulfB solution, SulfB-loaded HSA NPs and SulfB-loaded CS-HSA NPs in PBS.
Data displayed as mean $\pm$ SD; n=3.

Caco-2 experiments

Caco-2 cell line (human colorectal adenocarcinoma) was used as model of the nasal epithelium (Rassu et al., 2015; Rassu et al., 2017).

A first set of experiments was performed to select the optimum dilution of HSA NPs and CS-HSA NPs for further studies, i.e., permeation and uptake experiments. For this purpose, cell viability (MTS) assay was done. As reported in Figure 6, when Caco-2 cells were exposed for 4 h to different concentrations of HSA NPs and CS-HSA NPs (from dilution 1:40 to 1:10) the cell viability was not reduced. Further experiments demonstrated that when the cells were incubated with HSA NPs and CS-HSA NPs for 24 h diluted 10 times the cell viability was not significantly affected compared to the control group (Figure 6).

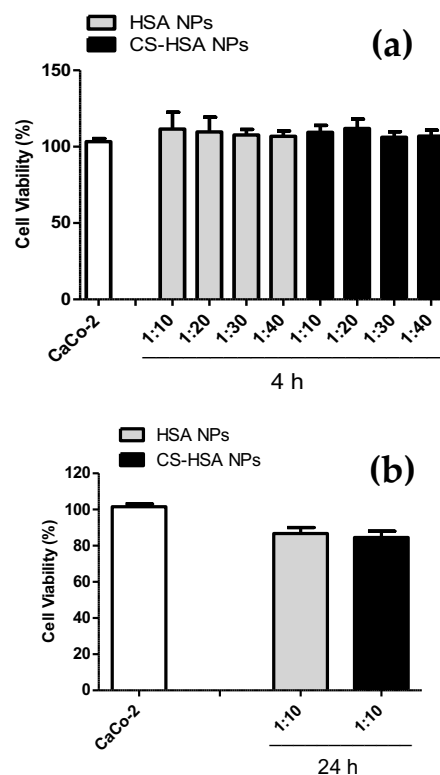


Figure 6. Caco-2 cell viability evaluated by MTS assay after exposure for 2 h (a) and 24 h (b) to HSA NPs and CS-HSA NPs.

Data are expressed as percentage of control (untreated group).

Values represent the mean $\pm$ SEM of at least three experiments performed in triplicate.

Since the formulations developed in this work are intended to be nasally administered, and the nasal epithelium represents the first barrier which they come into contact, these results indicate their biocompatibility, independently from the surface charge.

The permeation studies across Caco-2 monolayer revealed that the P_{app} values of HSA NPs and CS-HSA NPs were similar for the first 3 hours (Figure 7). This is probably related to the mucoadhesive characteristics that CS confers to the NPs and to the delayed drug release which occurs in the presence of CS-coating (Prego et al., 2005; Fonte et al., 2011; Fachel et al., 2018). However, after 4 h of incubation, the P_{app} value of CS-HSA NPs was higher (about double) than that the P_{app} value of HSA NPs (Figure 7).

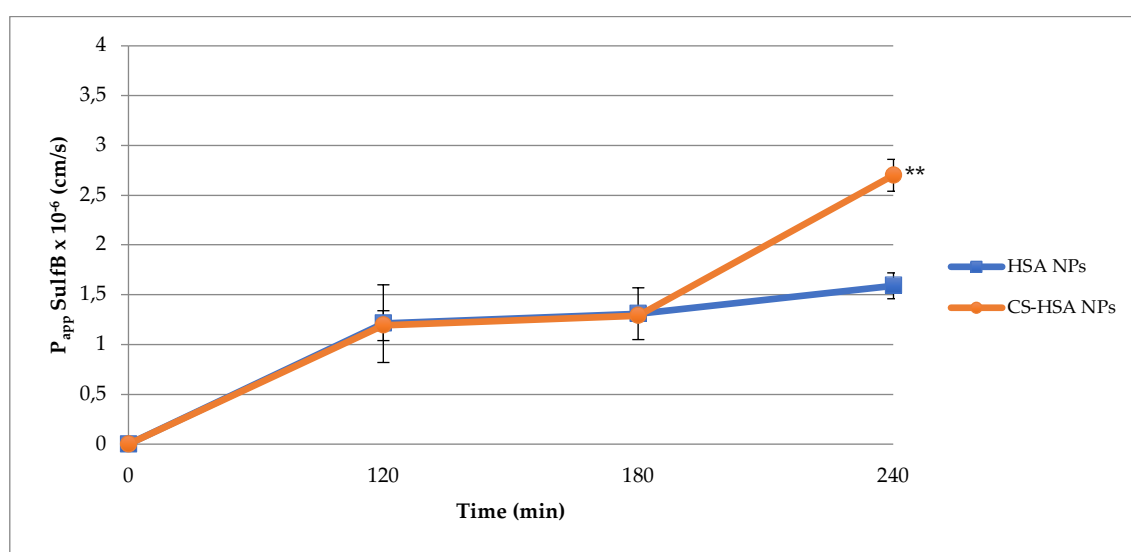


Figure 7. Permeation studies across Caco-2 cells.

Data displayed as mean $\pm$ SD; n=3. ** $p < 0.01$ vs HSA NPs. (ANOVA + Tukey's test).

This may occur due to the opening induced by CS of the tight junctions between the epithelial cells (Fonte et al., 2011). To confirm this, in the case of CS-HSA NPs, LY permeation slightly increased during the experiments, however, the percentage of permeation remained under 3% indicating that the integrity of the layer was maintained (Iacomino et al., 2013). Finally, the obtained P_{app} data are useful for *in vitro* prediction, as confirmed by the recovery values, which were above 80% in all experiments (Eigenmann et al., 2013).

To elucidate the endocytic uptake mechanism and to differentiate HSA NPs and CS-HSA NPs, the uptake experiments were carried out in the presence of sodium azide as energy depletion agent, chlorpromazine, a clathrin-dependent endocytosis inhibitor and indomethacin, as caveolin-dependent endocytosis inhibitor. As evidenced in Figure 8, the cellular uptake of HSA NPs and CS-HSA NPs was significantly inhibited in the presence of sodium azide, chlorpromazine and indomethacin with a greater effect of all the inhibitors in the case of uncoated NPs. These data indicated that both formulations, were internalized energy-dependently and the clathrin-mediated endocytosis resulted the main pathway involved in the uptake, consistent with the results obtained in other works with similar formulations (Byeon et al., 2016).

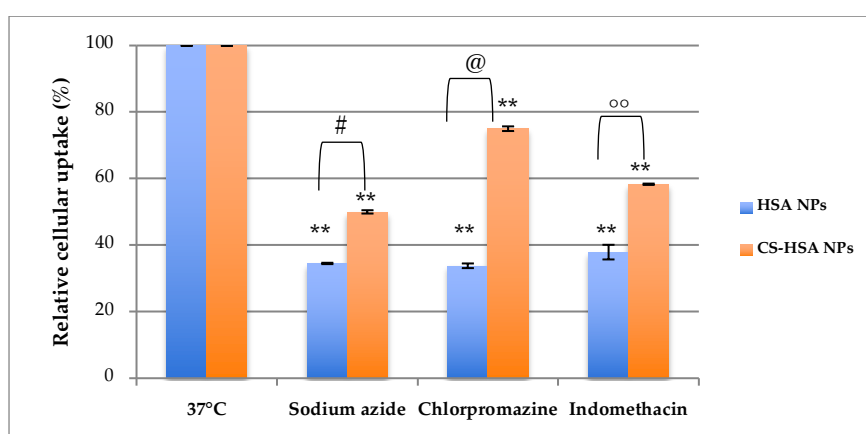


Figure 8. Effect of different inhibitors on Caco-2 cell internalization pathways of HSA NPs and CS-HSA NPs.

Data displayed as mean $\pm$ SD; n=3.

** $p < 0.01$ vs. 37°C; #, @, °° $p < 0.05$ vs. CS-HSA NPs. (ANOVA + Tukey's test).

hCMEC/D3 experiments

MTT and LDH assays were performed in hCMEC/D3 cell line to evaluate the effect of both NPs in cell viability and cytotoxicity, and permeability studies were also conducted in transwell devices using the same cell line. The *in vitro* cytotoxicity of the developed NPs was assessed by cell viability determination and membrane integrity evaluation using the hCMEC/D3 cell line in MTT and LDH assays, respectively (Figure 9). When cells were exposed to HSA NPs and CS-HSA NPs (from dilution 1:500, 1:100 and 1:10) for 2 and 24 h, no significant changes were observed in MTT metabolization, or LDH release when compared to cells exposed to the EBM-2 medium alone, indicating that HSA NPs and CS-HSA NPs affected neither the metabolic activity of the cells nor the membrane integrity at these time points.

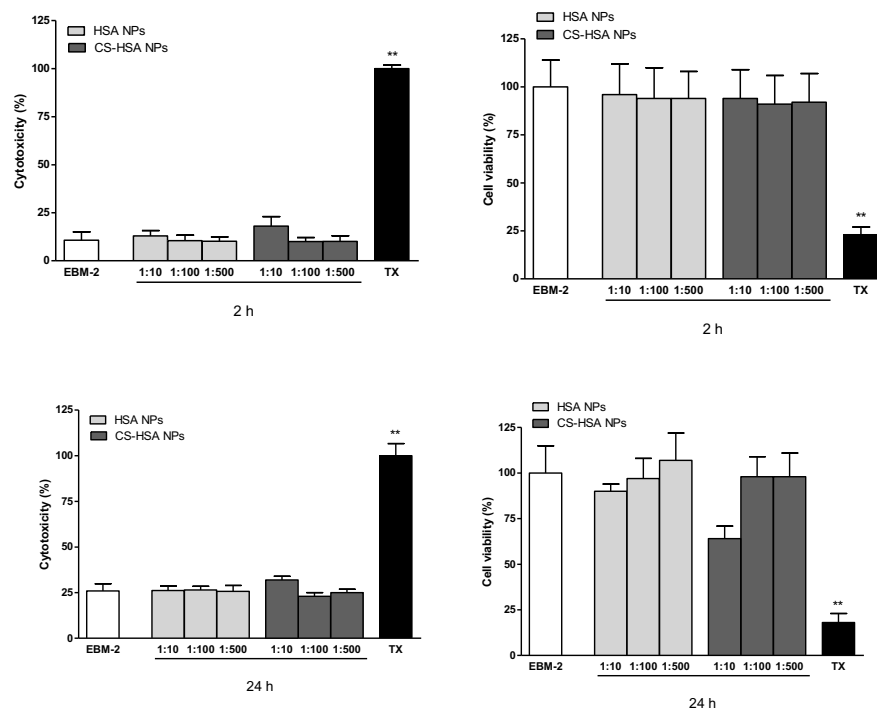


Figure 9. hCMEC/D3 cell viability evaluated by MTT assay (right panel) and cytotoxicity by LDH assay (left panel) when exposed for 2 h (a), and 24 h (b) to HSA NPs and CS-HSA NPs. Data are expressed as percentage of control (EBM-2 medium) and Triton-X which represent, respectively, the maximum cell viability and cell cytotoxicity.

Values represent the mean $\pm$ SEM of at least three experiments performed in triplicate.

** $p < 0.01$ vs. EBM-2 alone.

hCMEC/D3 brain microvascular endothelial cell line is a model of human BBB utilized to study the drug transport mechanisms (Eigenmann et al., 2013; Guccione et al., 2017). The cells retain the expression of most transporters and receptors expressed *in vivo* in the human BBB, therefore permeability studies were performed to predict the permeability of SulfB-HSA NPs and SulfB-CS-HSA NPs across the BBB. NaF was used as negative control and its P_{app} was determined to monitor the integrity of the cell layer. The integrity of the cell layer was also assessed by phase-contrast microscopy (Guccione et al., 2017).

The permeation studies across hCMEC/D3 monolayer revealed that the P_{app} values of SulfB-CS-HSA NPs is higher than SulfB-HSA NPs at 1 and 2 hours (Figure 10). This is probably related to the mechanism of action of chitosan which induces a reversible transient change of the tight junctions. However, after 3 h of incubation, the P_{app} value of SulfB-CS-HSA NPs and SulfB-HSA NPs was similar.

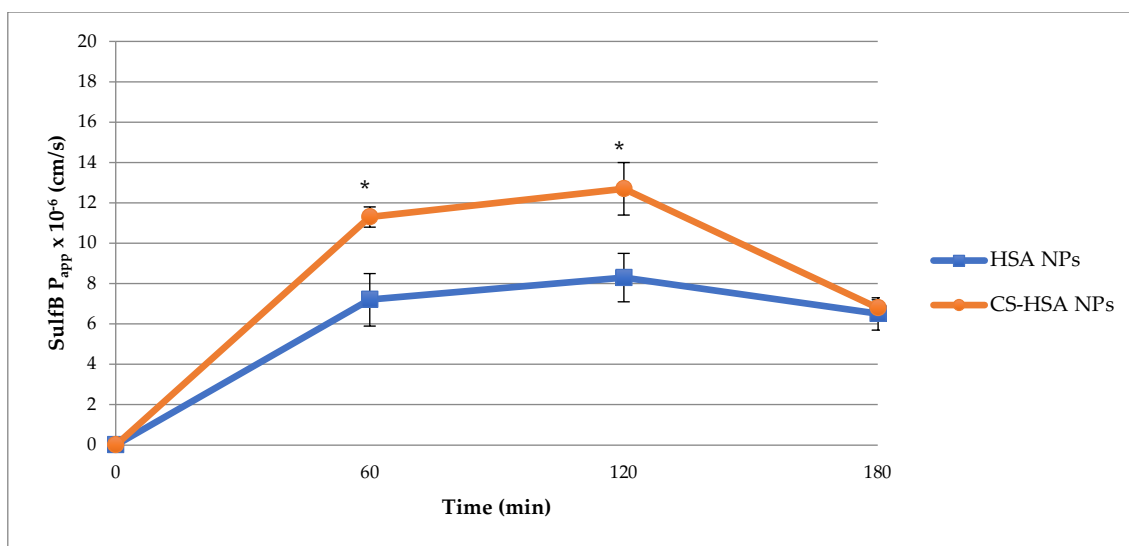


Figure 10. Permeation studies across hCMEC/D3 cells.

Data displayed as mean $\pm$ SD; n=3.

* $p < 0.05$ vs HSA NPs. (ANOVA + Tukey's test).

To elucidate the endocytic uptake mechanism and to differentiate HSA NPs and CS-HSA NPs, the uptake experiments were carried out in the presence of sodium azide as energy depletion agent, chlorpromazine, a clathrin-dependent endocytosis inhibitor and indomethacin, as caveolin-dependent endocytosis inhibitor. As evidenced in Figure 11, the cellular uptake of HSA NPs and CS-HSA NPs was significantly inhibited in the presence of sodium azide, chlorpromazine and indomethacin with a greater effect of all the inhibitors in the case of uncoated NPs. These data indicated that both formulations, were internalized energy-dependently and the clathrin-mediated endocytosis resulted the main pathway involved in this uptake.

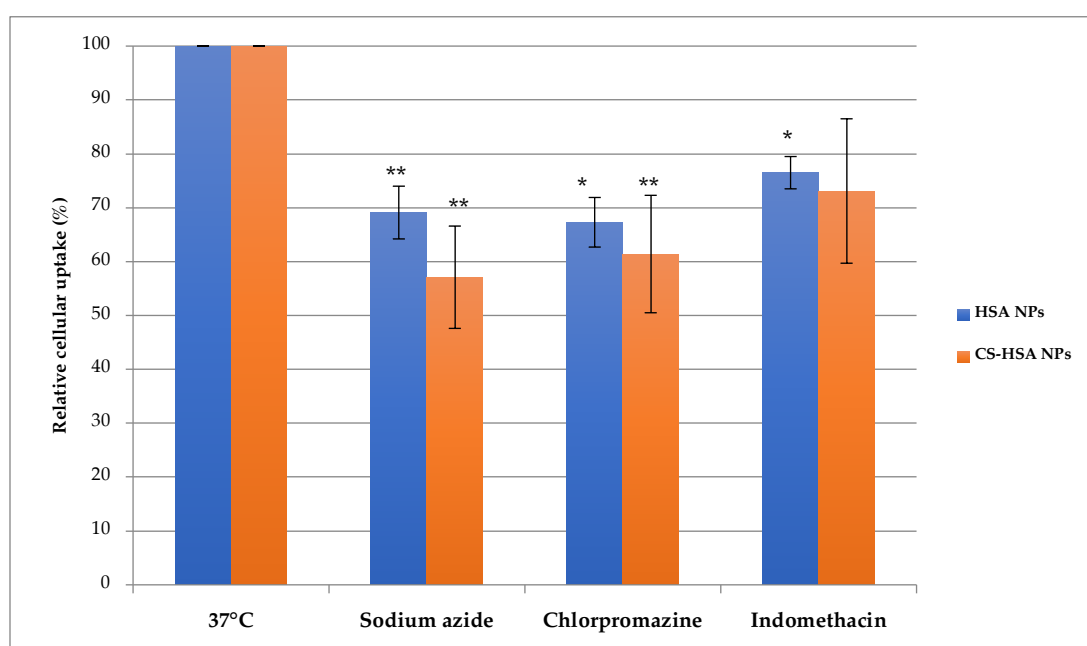


Figure 11. Effect of different inhibitors on hCMEC/d3 cell internalization pathways of HSA NPs and CS-HSA NPs.

Data displayed as mean $\pm$ SD; n=3.

** $p < 0.01$ vs. 37°C; * $p < 0.05$ vs. 37°C. (ANOVA + Tukey's test).

Ex vivo transport experiments across rabbit nasal mucosa

The *ex vivo* permeation of SulfB from HSA NPs, CS-HSA NPs and aqueous solution was studied by using rabbit nasal mucosa as biological barrier (Russo et al., 2006; Bortolotti et al., 2009; Giuliani et al., 2018). As reported in Figure 12, the maximum permeation of SulfB in 24 h was found to be 83.7% for CS-HSA NPs, 68.9% for HSA NPs and 45.5% in the case of the aqueous solution. The higher permeation of SulfB, when loaded into CS-HSA NPs could be due to the better permeation enhancing activity of CS, in particular to the interaction of positively charged amino groups of CS with negatively charged cell membranes and to the opening of the tight junction of the mucosal epithelial cells. On the other hand, the probable reason for the lesser permeation of the free-SulfB in comparison to SulfB loaded into HSA NPs and CS-HSA NPs could be due to the hydrophilic nature of drug (Bari et al., 2015).

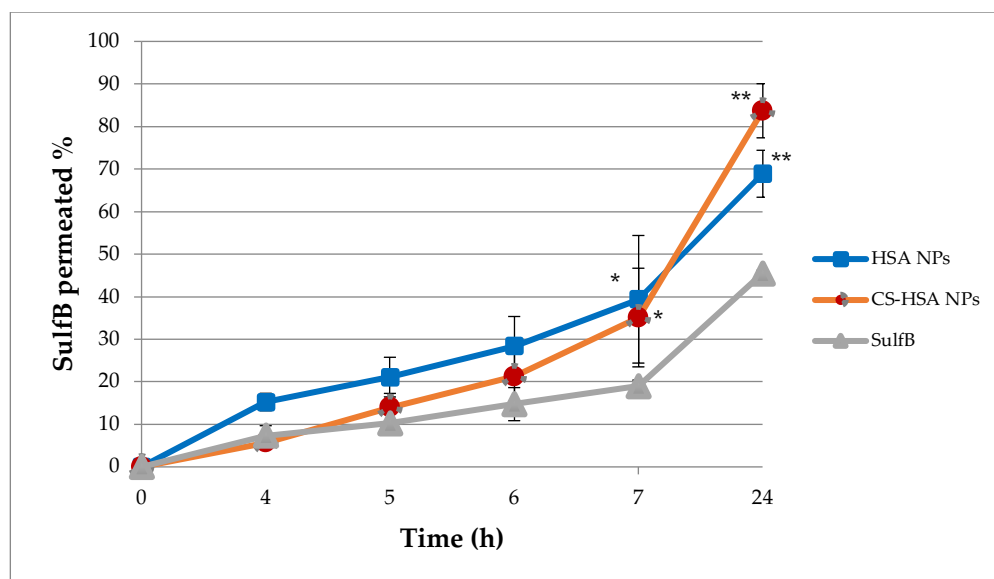


Figure 12. *Ex-vivo* permeation studies of SulfB solution, SulfB-loaded HSA NPs and SulfB-loaded CS-HSA NPs conjugates through rabbit nasal mucosa.

Data displayed as mean $\pm$ SD; n=3.

** $p < 0.01$ vs. SulfB solution; * $p < 0.05$ vs. SulfB solution. (ANOVA + Tukey's test).

Besides, the permeation rate of SulfB loaded into CS-HSA NPs resulted $16.7 \pm 1.3 \times 10^{-6}$ cm/s, while the permeation rates of HSA NPs and free-SulfB resulted $13.8 \pm 1.1 \times 10^{-6}$ cm/s and $9.1 \pm 0.1 \times 10^{-6}$ cm/s, respectively. Larger permeation coefficient is expected to correlate with enhanced absorption (G. Chen et al., 2018). The obtained data are suitable for an accurate prediction since the mass balance values were 98.9 ± 1.6 % for HSA NPs and 99.6 ± 0.6 % for CS-HSA NPs.

Looking more closely the permeation profiles in Figure 12, it could be affirmed that the presence of CS-coating on the surface of HSA NPs determined a slower amount of SulfB permeated in particular during the first 6 h, in comparison to uncoated NPs.

This result is probably due to the mucoadhesive properties of CS-HSA NPs and to the time required for CS hydration and swelling, which could result in an extended time of action, in accordance with the results obtained by the *in vitro* release studies and Caco-2 experiments (Prego et al., 2005; Singh & Lillard, 2009; Fonte et al., 2011; Fachel et al., 2018; Raj et al., 2018).

As evidence of the mucoadhesive properties of CS-HSA NPs respect to HSA NPs, the duration of the first set of experiments was set to 4 h. As hypothesized, concerning the amount of SulfB recovered into the rabbit mucosa nasal after 4 h, CS-HSA NPs exhibited higher SulfB retention (6.7%) respect to HSA NPs (1.7%). These results demonstrated the strong interaction between CS and the nasal mucosa.

CONCLUSIONS

In this study, the influence of CS-coating on HSA NPs for nasal delivery was investigated for the first time.

Based on the obtained results concerning physical and chemical parameters, the developed formulations resulted suitable for nasal application. The freeze-drying process was optimized, and both HSA NPs and CS-HSA NPs showed excellent storage stability properties as suspensions, at 4°C, and as freeze-dried products. The functionalization coating with CS was confirmed by the difference in the ζ -potential value and also by TEM analysis. Besides, the mucoadhesive properties of CS and its ability to modulate the release of the entrapped molecule were proved by the turbidimetric and the indirect method and performing *in vitro* release studies. Caco-2 and hCMEC/D3 cell experiments (selected as model of nasal epithelium and BBB, respectively) evidenced the safety of the NPs and the potential of CS to increase the drug permeation. Both formulations were internalized into these cells energy-dependently and the clathrin-mediated endocytosis resulted the main pathway involved in this process. *Ex-vivo* studies demonstrated a strong interaction between CS and the nasal mucosa and the higher penetrating potential of CSA-HSA NPs respect to uncoated NPs. Consequently, the present research proposed a novel and promising strategy based on HSA NPs surface modification for the NTB delivery of many bioactives.

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. Caco-2 cells studies were performed in collaboration with Dr. Cristina Luceri, Dr. Lorenzo Cinci and Dr. Mario D'Ambrosio (NEUROFARBA, Department of Neurosciences, Psychology, Drug Research and Child Health, Section of pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).

. hCMEC/D3 cells studies were performed in collaboration with Prof. Domenico E. Pellegrini-Giampietro and Dr. Elisa Landucci (Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy).

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