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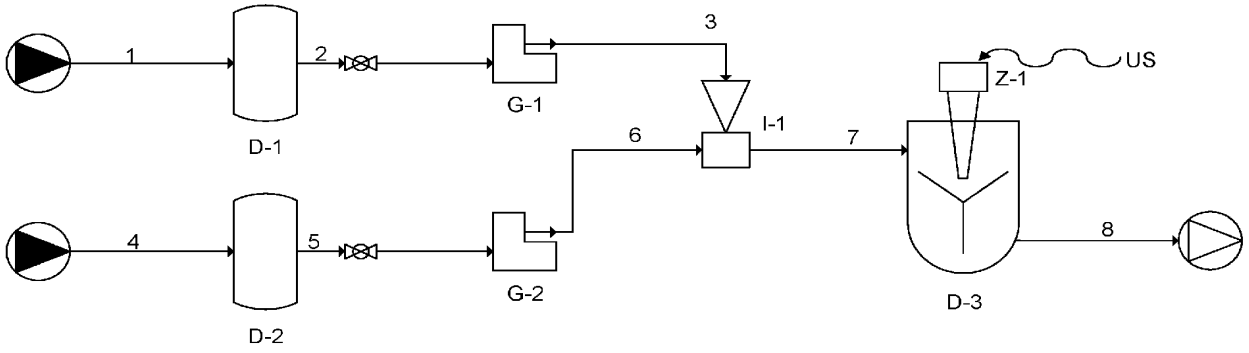

Fig. 1

(57) Abstract: The invention relates to a continuous or semi-continuous, high productivity process for obtaining nanoliposomes containing iron (II) combined with a reducing agent capable of increasing the stability and bioavailability of iron (II), such as ascorbic acid or a salt thereof, which process is based on a high productivity "simil-microfluidic" technique for nanoliposome formation, coupled with an ultrasound treatment for the homogenization of the nanoliposomes obtained. The invention also concerns the nanoliposomes obtained by means of the described process, and the food and nutraceutical products enriched with iron(II), in which enrichment is accomplished by using said nanoliposomes as a source of bioavailable iron.

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PROCESS FOR PREPARING NANOLIPOSOMES COMPRISING MICRONUTRIENTS AND FOOD PRODUCTS COMPRISING SAID NANOLIPOSOMES

DESCRIPTION

Field of the invention

The present invention relates to a process for the production of nanoliposomal vectors encapsulating highly bioavailable iron with a continuous process, as well as the nanoliposomes thus obtained. More particularly, the invention relates to a continuous, and therefore to highly productive, process for obtaining nanoliposomes containing iron(II) combined with a reducing agent capable of increasing the stability and bioavailability of iron(II), which process is based on a high productivity "simil-microfluidic" technique for nanoliposome formation, coupled with an ultrasound treatment for the homogenization of the nanoliposomes obtained.

Background of the invention

As is known, the encapsulation of active pharmaceutical, nutraceutical or cosmetic ingredients in micro- or nanoparticles is an increasingly widespread method to protect the active ingredient molecules from the external environment, to increase their bioavailability, to mask any undesirable properties thereof, to direct their release into the tissues where it is desired that they exert their action and/or to control the moment or the duration of such action. Said micro- or nanoparticles, particularly, may be polymeric or lipidic micro- or nanoparticles, and the latter, in turn, include micro- or nanospheres, i.e. solid lipid particles, and liposomes, which can be micrometric or nano-sized as well.

Unlike solid lipid micro-or nanoparticles, which are made entirely of lipid materials in which a liposoluble active ingredient is carried, liposomes are closed vesicular structures, consisting of one or more double phospholipid layers, which are formed when membrane phospholipids, such as phosphatidylcholine or cholesterol, are dispersed in an excess of water. Unlike micelles, in liposomes both the external and internal environment are aqueous.

Among the several applications of commercially exploited liposomal carriers, there is the release of vitamins and micronutrients such as iron in nutraceuticals products, food supplements and fortified foods.

In general, a number of techniques have been used to date to produce iron delivery systems for nutraceutical purposes, and there are several corresponding commercial products on the market. It is known in the literature that the most directly bioavailable form of iron is the form with oxidation number 2 (i.e. Fe(II), which occurs in ferrous salts, such as ferrous sulphate) (Allen, L., et al., *Guidelines on food fortification with micronutrients* 2006: World Health Organization, food and Agriculture Organization of the United Nations, see Table 5.1, in particular). On the other hand, it has been found that once added to the final product, the Fe(II) form tends to oxidize, losing its bioavailability and imparting an unpleasant taste to the product.

It is also known that in order to further increase the bioavailability of iron and enhance its assimilation it is necessary to dose it in combination with a carrier molecule capable of protecting it from oxidation, such as ascorbic acid or a salt thereof, in a weight ratio ascorbic acid:Fe = 6:1 (Hurrell, R., *How to Ensure Adequate Iron Absorption from Iron-fortified Food*, Nutrition Reviews, 2002. **60**: S7-S15). This recommendation is also confirmed by the guidelines of the World Health Organization (cited above).

Although part of the current iron-based dosage forms have found an industrial application, the possibility of overcoming operational disadvantages and, possibly, increasing the quality of products is constantly being studied. Among the products already on the market, for example, the liposomal ferric pyrophosphate (sold under the trade name 'Sideral') contains iron carried in liposomes, but in this case the iron is in the oxidation state +3 (Fe(III)).

In the light of the above, it is evident that a biocompatible structure such as that of liposomes, which is able to protect the iron and the molecule associated with it by preserving the iron in the oxidation state +2 (Fe(II)), represents a preferential choice for the production of effective drug delivery systems for nutraceutical products and fortified foods.

In fact, the liposomal carriers are structures which can carry iron

through different administration routes, first of all through the oral and the injection route. Currently, liposomes are among the most used carriers, because they offer great advantages both in terms of biocompatibility and in terms of preparative versatility. Their biocompatibility is ensured by their own composition and structure, which mimics the cell wall and therefore makes them easily absorbed by the cells, while from a preparatory point of view, they offer the advantage of having their size set on micro- or nano-scale depending on the procedure adopted for their production. Moreover, they have the additional advantage of being capable to be functionalized.

However, it is to be considered that, while on a laboratory scale the complexity of excellent preparation techniques is not an interesting factor for the researcher, on an industrial scale simple, possibly continuous techniques which guarantee excellent products in terms of quality are obviously highly desired.

Among the techniques most applied or studied by researchers for the preparation of liposomal iron delivery systems (both as Fe(II) and Fe(III)) the following ones may be cited:

- production by *freeze-thawing* (FT), with cooling and heating cycles;
- production by controlled hydration of lipid films or *thin-film hydration* (TF);
- production by injection of solvent, in particular ethanol, *ethanol injection method* (EI);
- high pressure homogenization *in microfluidizer* (MF);
- production by sonication and evaporation of the solvent (*reverse-phase evaporation*, REV).

As described in the US patent 5534268 (De Paoli et al., *Liposomes containing bioavailable iron(II) and processes to obtain them*) the production technique by *freeze-thawing*, FT, provides for the formation of liposomal vesicles in an aqueous homogenization charge containing phospholipids (lecithin phosphoglycerides), treated in a turbine homogenizer to obtain a homogeneous suspension, and then subjected to alternating cycles of freezing (-10°C) and thawing (25°C). The inclusion of iron occurs by the addition of an aqueous

solution of iron(II) (ferrous sulphate, ferrous lactate or ferrous citrate) and proceeding again to several freezing and thawing cycles.

The *thin film hydration* technique, TF, which has been the first mechanical technique to be developed and is still the most widespread, involves the formation of lipid films starting from phospholipids solubilized in organic solvents such as chloroform or methanol, which are then removed by evaporation under vacuum in a rotary evaporator, leaving a thin lipid film on the wall of the evaporator. Subsequently, the lipid film is hydrated, under adequate stirring, with an aqueous solution containing iron(II). This method is described as an alternative embodiment for the production of liposomes loaded with ferrous salts in the previously mentioned patent US 5534268, as well as in the review of Xia and Xu (Xia, S. and Xu, S., *Ferrous sulphate liposomes: preparation, stability and application in fluid milk*, Food Research International, 2005. **38**(3): pages 289-296 - see, in particular, page 291).

In the preparation of liposomes by solvent injection there are two miscible phases, one of which containing the dissolved lipids, which phases are mixed together to give the aggregation of lipids which forms the liposomes. In particular, in the *ethanol injection* (EI) technique, lipids are dissolved in ethanol and then the solution is rapidly injected into the aqueous phase. After the liposomes formation, generally at 60-70°C, the solvent is removed by evaporation under reduced pressure (Kremer, J. et al., *Vesicles of variable diameter prepared by a modified injection method.*) Biochemistry, 1977. **16**, 3932 - 3935).

The *microfluidic technique* (MF), suggested by the patent EP2338478 (Protiva Biotherapeutics Inc., *Method for producing liposomes*), involves the contact of a stream of dissolved lipid phase in an organic solvent and an aqueous stream containing the iron through their passage in a microfluidic homogenizer equipped with microchannels, operating at high pressures (Pradhan, P., et al., *A Facile Microfluidic Method for Production of Liposomes*. Anti Cancer Research, 2008. **8**: p. 943-948; Kosaraju, S.L et al., *Liposomal Delivery Systems for Encapsulation of Ferrous Sulphate: Preparation and Characterization*. Journal of Liposome Research, 2006. **16**(4): p. 347-358). It should

be considered that the microfluidic technique allows a continuous production and an accurate control of the liposomes size, but is affected, however, by the problem of the scale-up necessary to switch from experimental to industrial production, given that the volumetric flow rates involved in microfluidic applications are extremely low and therefore their potentiality is extremely low (Yu, B., et al., *Microfluidic methods for production of liposomes*. Methods Enzymol., 2009. **465**:129-141; Jahn, A., et al., *Microfluidic directed formation of liposomes of controlled size*. Langmuir. 2007. **23**:6289-6293).

The *reverse-phase evaporation* (REV) technique involves the formation of lipid films starting from phospholipids solubilized in organic solvents (usually diethyl ether, isopropyl ether or a mixture of isopropyl ether and chloroform) and an aqueous buffer. The resulting biphasic system is sonicated until a homogeneous emulsion is obtained, and the solvent is then removed by evaporation under reduced pressure nitrogen atmosphere. Subsequently, the lipid film is re-dissolved under vacuum conditions and then it is added with the iron(II) contained in an aqueous phase (Xia, S. and Xu, S., already cited).

The aforementioned techniques have, as their main disadvantage, the feature of being discontinuous techniques (operating in batches), with laborious process steps involving the continuous manipulation of the prepared suspensions. Furthermore, it is not negligible the use of solvents which, although removed by evaporation steps, may constitute final contaminants. Operating conditions such as low temperatures, low pressures, high pressures and the use of inert atmospheres make the mentioned techniques burdensome, also from an energetic point of view. Furthermore, the encapsulation efficiencies (EE) are inherently not very high (57%<EE%<85%).

Finally, it should be noted that not always the mentioned techniques allow to obtain nanoscale products. Liposomes of nanometric dimensions are to be preferred to the micrometric systems normally obtained, because they have a larger surface area of interface, they allow a better dispersion in the delivery systems, as well as a better bioavailability and a more controlled release of the iron, thanks to a prolonged retention time in the physiological environments where the absorption of iron occurs, as the intestine (Singh, H.,

Nanotechnology Applications in Functional Foods; Opportunities and Challenges. Preventive nutrition and food science, 2016. **21**(1): p. 1; Huang, Q., *Nanotechnology in the food, beverage and nutraceutical industries*. 2012: Elsevier).

In the light of the aforementioned prior art, the present invention is thus aimed at providing stable liposomal carriers, encapsulating with high efficiency iron in a highly bioavailable form (i.e. in the form of ferrous salts) coupled with an antioxidant in proportions which guarantee the maximum bioavailability and chemically stability, and which can be produced with a continuous technique with high productivity.

Summary of the invention

In the frame of the studies connected with the present invention it has been considered that most of the cited methods are characterized by a considerable energy demand, long processing times and low productivity. In particular, the most used techniques, such as ethanol injection, are laboratory-scale methods characterized by discontinuous batch processes. On the other hand, the microfluidic techniques described in the recent literature represent relatively new solutions which allow the production of liposomes on a nanometric scale and give the possibility of continuously producing small unilamellar liposomes (SUVs) with precise control over their dimensional characteristics, by modulating the flows involved on a micrometric scale. However, the implementation of these techniques is burdened by high costs of microfabrication of the necessary devices, and also by low productivity.

In order to overcome these limitations, according to the present invention the microfluidic techniques have been transposed to a millimeter scale, in particular starting from the work of Pradhan et al. (cited above), wherein a microfluidic device with a volumetric plunger pump consisting of a syringe was used to produce liposomes. According to the invention, a simple device suitable for a mass production of nanoliposomes has been devised and set up, which allows to overcome the limits imposed by the prior art syringe device. This device uses two volumetric pumps to supply, at volumetric flows in the

order of mL/s, respectively a lipid stream dissolved in an organic solvent and an aqueous stream containing the iron at a point of contact between the two streams. Said point of contact, according to an exemplary embodiment, consists of a needle having a 0.6 mm internal diameter (lipid stream) inserted into a silicone tube with an internal diameter of 3 mm and a length of 185 mm, which constitutes the extension of the aqueous stream tube, within which the formation of the nanoliposomes takes place.

The translation of microfluidic techniques to the millimeter scale is itself an innovation of the processes available in the scientific literature and in practical applications. In fact, microfluidic techniques are characterized by sub-millimetric channel sizes (up to 500 μm) and flow rates in the range of μL/s: under these conditions the flows occur in laminar regime with extremely low Reynolds numbers (less than 100, or even less than 10: an intubated flow is laminar for Reynolds numbers lower than 2100). For example, in Jahn et al. (Jahn, A., et al., *Microfluidic directed formation of liposomes of controlled size*. Langmuir, 2007, **23**:6289-6293) the channels are 100 μm deep and 64 μm wide, the maximum volumetric flow rate is 150 μL/min. Under these conditions, assuming the water properties for the fluids, the Reynolds number is about 30, i.e. the flow regime is definitely laminar. In conditions of mass flow or microfluidic conditions, the dimensions of the channels are greater than one centimeter, the flow rates are of the order of L/s: in these conditions the flows almost always take place in turbulent flow regime, with high Reynolds numbers, even above 100,000.

The millimetric scale proposed in the present invention, referred herein as simil-microfluidic, is intermediate between the microfluidic and the macrofluidic scale. The dimensions of the channels are in the order of millimeters, the flow rates in the order of mL/s: in these conditions the flows occur mainly in the laminar flow regime, with low Reynolds numbers, in the order of 1000. For example, in the application of the present invention, using a tube with an internal diameter of 3 mm, a volumetric flow rate of 1 mL/s and assuming the physical characteristics of water for the fluid, a Reynolds number equal to 425 is obtained, i.e. a laminar flow regime. It should also be noted that in

microfluidic applications the most important hydrodynamic forces are the capillary forces and the surface tension, while mass transfer takes place mainly with a molecular mechanism (diffusion). In macrofluidic applications the most relevant hydrodynamic forces are the inertial forces and the viscous forces, while the mass transfer takes place mainly with a convective mechanism. In the applications according to the present invention (i.e. simil-micro-fluidic applications) the most relevant hydrodynamic forces are the same as for the macrofluidic applications (inertial forces and viscous forces), while the mass transfer can take place with molecular and/or convective mechanism.

Moreover, since the exchange surface increases with the square of the characteristic length while the volume increases with the cube of the characteristic length and the mass transfer phenomena are dominated by the surface/volume ratio, the reduction of a factor 10 in the characteristic length increases by a factor of 10 the surface/volume ratio. Finally, the equipment and the devices that can be used for a simil-microfluidic process are more common and less expensive than those required for microfluidics, and the production rates involved in a simil-microfluidic process are higher than those of microfluidics. From all these considerations there follows that simil-microfluidics can be an alternative approach to microfluidics and macrofluidics, depending on which need it is desired to reconcile. In the present invention, the simil-microfluidic approach is preferred because it allows to obtain significant flow rates (and therefore significant productivity) while allowing to work with the high control of the operating conditions consequent to the laminar flow regime.

The process of the invention therefore essentially produces a contact between two flows, on one side phospholipids, preferably consisting of phosphatidylcholine and cholesterol in an alcoholic solution and, on the other side, an aqueous solution containing the ferrous salt (Fe(II)) in combination with a reducing and stabilizing compound, such as ascorbic acid or a salt thereof, inside a tubular device in which interdiffusion phenomena between the two phases cause the formation of lipid vesicles of nanometric size, containing the Fe(II) salt in combination with the reducing compound therein.

According to the invention, the dosage formulation of Fe(II) is selected

so as to achieve an optimal ratio with the reducing/stabilizing compound, with a ratio such as to maximize the bioavailability, preferably, in the case of ascorbic acid, equal to iron:ascorbic acid = 1:2 by moles, which equals 1: 6 as a weight ratio. Therefore, in the proposed invention the iron is used in a very bioavailable form, being in the state of oxidation (II, -ous) and being combined with a molecule which promotes its bioavailability, ascorbic acid or a salt thereof, in the ratios which were found to correspond to the maximum increase in bioavailability. The oxidability of iron(II) and its unpleasant taste are also minimized by encapsulation of the ferrous salt and ascorbic acid into nanoliposomes produced using phospholipids and cholesterol.

The cited ingredients are processed in the production method according to the invention in a continuous or semi-continuous manner, to obtain said nanoliposomes. The latter are produced with an average size of less than, or equal to, 100 nm and with a polydispersity index of less than 0.5. The proposed production method, which derives from the microfluidic approaches but does not use micrometric size equipment, consists in the injection of an alcoholic solution of membrane lipids into a channel in which an aqueous solution of the iron salt and ascorbic acid or salt thereof flows. The flow rates and dimensions of the pipes are selected in such a way that the fluids always flow under the laminar flow regime.

As already noted, the nanoliposomes are formed at the point of contact between the two solutions, after which the resulting hydroalcoholic solution carrying the nanoliposomes in suspension is directed to a section where it undergoes an ultrasonic homogenization, which reduces and uniforms the average size of the nanoliposomes, thus reducing the polydispersity of said nanoliposomes.

It should be considered that in the proposed invention the non-micrometric scale (simil-microfluidic technique) allows high productivity, differently from the microfluidic technological solutions (Yu, B., et al., *Microfluidic methods for production of liposomes*. Methods Enzymol., 2009. **465**: 129-141), while maintaining the possibility of an accurate control of the size and polydispersity of the liposomes. This diverges from processes which, being

based on mass mixing (macrofluidics), require further membrane extrusion and filtration steps (US patent 2015/174070A1, Cheng, ML & Huang, YK, *Liposome suspensions, method for preparing the same, and application thereof*). Moreover, the sequence of the two steps of injection and ultrasonic homogenization allows to take advantage of the accurate control in the production phase of the liposomes, which is typical of an injection under "orderly" (laminar) flow conditions and, eventually, to reduce the dimensions of the products thanks to the action of ultrasounds. The application of ultrasounds during the injection phase, on the contrary, frustrates the positive effects of the laminar flow since the agitation due to the ultrasounds creates chaotic flows in the fluids. Although it is possible to obtain nanoliposomes by applying ultrasounds during the injection step (Huang, X., et al., *Ultrasound-enhanced microfluidic synthesis of liposomes*, Anticancer Research 2010. **30**: 463-466), in that case it is reasonable to assume that the reduction in size is due entirely to the action of the ultrasounds, and therefore the process would be more energy-consuming than the sequential approach, i.e. injection followed by homogenization (Barba, A.A., et al., *Ultrasonic energy in liposome production: process modelling and size calculation*, Soft Matter. 2014. **10**:2574-2581).

Detailed description of the invention

Therefore, the present invention specifically provides a process for the preparation of nanoliposomes for the administration of iron and other micronutrients in nutraceutical or fortified food products, which process comprises the following operations:

a) producing a first alcoholic solution of phospholipids and cholesterol at a concentration of from 0.1 to 10 mg/mL, preferably 5 mg/mL;

b) producing a second aqueous solution containing iron(II) salts, such as ferrous sulphate, ferrous citrate and ferrous lactate, and possibly salts of other micronutrients selected from the group of metal elements and their mixtures, together with a reducing compound such as ascorbic acid or a salt thereof;

c) pumping said alcoholic and aqueous solutions through two volumet-

ric pumps in flow rates of the order of mL/s and in a respective volume flow rate ratio of from 5:1 to 50:1, preferably 10:1;

d) contacting the flows consisting of said two solutions by making them pass through a device operating in simil-microfluidic regime, i.e. a device which effects formation of nanoliposomes by injection of said first solution stream into said second solution stream through a duct, for instance a hollow needle, of internal diameter below 1 mm, the duct of said second solution having an internal diameter of 2-5 mm and a length after the injection point of 150-200 mm;

e) subjecting to an ultrasound treatment the nanoliposomes suspension obtained from the previous operation, to reduce the size of the nanoliposomes produced and limit their polydispersity,

the nanoliposomes thus obtained having an average size not higher than 100 nm and a polydispersity index of less than 0.5.

Further characteristics of the nanoliposomes according to the invention and of the process for their production are set forth in the subsequent claims.

According to the invention, the alcoholic solution must be prepared so as to contain the lipids necessary for the production of the nanoliposomes. Preferred lipids are a phospholipid such as lecithin (i.e. phosphatidylcholine, PC) and a membrane lipid such as cholesterol (COL). The most suitable molar ratio between these two lipids is PC:COL = 2.5:1, which replicates the cell membrane compositions and thus promotes the fusogenic actions and the transfection.

The lipids concentration in the alcoholic solution is a process parameter, and as such it can be varied to change the characteristics of the nanoliposomes obtained, such as the average size. The reference of 470 mg of phosphatidylcholine (PC) and 94 mg of cholesterol (COL) in 10 mL of ethyl alcohol can be used, i.e. 0.6 mmol of PC and 0.24 mmol of COL in 10 mL of ethyl alcohol.

The aqueous solution must be prepared using an iron(II) salt, for example ferrous sulphate, and ascorbic acid or a salt thereof in order to obtain a molar ratio of iron to ascorbic acid of 1:2, corresponding to a weight ratio of

iron to ascorbic acid of 1:6. Under these conditions, the iron uptake in adults and children is increased by 2 to 3 times compared to the uptake of equal amounts of iron not combined with ascorbic acid.

The iron concentration in the aqueous solution is a process parameter, correlated to the ratio of the metered iron to the total of components other than the dosed solvents, i.e. the ratio of iron to the sum of iron, ascorbic acid, lecithin and cholesterol. This sum is defined as the total of the components. To determine the ratio iron/total of components it is necessary to take into account the feeding ratio of the two flow rates of alcoholic solution and aqueous solution to the injection device. As a reference value for the ratio iron/total of components, 0.06 can be used, i.e. 6% of iron on the total of the components.

In more detail, the process for producing the nanoliposomes of the invention may be described as comprising the following unit operations:

A. Preparation and storage of the aqueous solution, in a first stirred vessel connected to the next pumping section;

B. Preparation and storage of the alcohol solution, in a second stirred vessel connected to the next pumping section;

C. Pumping of the two solutions using peristaltic pumps or other volumetric devices. Peristaltic pumps are preferred in view of the negligible interaction with pumped fluids and for their easy process scalability;

D. Injection of the alcohol solution by means of a needle into the tube where the aqueous solution flows. The internal diameters of the pipes and the flow rates must ensure that all flows are in laminar regime, the length of the piping of the aqueous solution must be such as to exclude entrance effects on the development of the speed profile. The fluid dynamic conditions replicate those observed in the microfluidics field. In the proposed process, however, the systems have sizes on a millimetric scale, thus avoiding the difficulties and costs of implementation, and the energy consumption typical of the microfluidic systems. As reference values, an internal needle diameter of 0.6 mm and an internal tube diameter of 3.0 mm for the aqueous solution may be assumed. This choice corresponds to a ratio of the two diameters equal to 1:5,

which is therefore a reference value for this parameter. The ratio of the flow rate of the aqueous solution to the flow rate of the alcoholic solution may vary between 5 and 100, with values of greatest interest being between 10 and 40.

E. Ultrasonic homogenization of the hydroalcoholic solution obtained in the previous item C). Homogenization by sonication can be carried in batch by using cycles consisting of phases of ultrasounds application alternating with cooling steps in semi-continuous productions on a small scale, or continuously in a refrigerated sonication cell.

The nanoliposomes suspension thus obtained is then stored for purposes of characterization and/or use.

In one embodiment, the process according to the invention allowed to produce stable nanoliposomes loaded with iron(II) through the simil-microfluidic apparatus described using a ferrous sulphate/total of components ratio equal to 0.01 by weight and obtaining nanoliposomes with an average diameter of 76 nm, with 97% of encapsulation efficiency (EE), and with a polydispersity index of 0.37.

According to a further aspect thereof, the present invention also relates to food products and nutraceuticals enriched with iron(II), in which enrichment is achieved by using nanoliposomes obtained from the process described herein as a source of bioavailable iron.

Brief description of the figures

The specific featuers of the invention, as well as the advantages of the same, will be more evident with reference to the accompanying drawings, in which:

Figure 1 shows an exemplary plant scheme for realizing the process of the present invention;

Figure 2 shows in a histogram form the average diameters of the nanoliposomes produced (diagram A) and their polydispersity index (diagram B), before and after sonication, as the volume flow rate ratio of the aqueous solution to the alcohol solution;

Figure 3 shows in a histogram form the average diameters of the nanoliposomes produced (diagram A) and their polydispersity index (diagram B), before and after sonication, plotted vs. the variation of the phosphatidylcholine concentration (PC) expected in the hydroalcoholic solution after injection; and

Figure 4 shows the progression of the iron contained in the nanoliposomes according to the invention and released in deionized water as a function of time.

EXAMPLES

Some specific embodiments of the production process according to the invention are described below, purely by way of non-limiting examples, together with the results of the clinical experimentation carried out.

Plant scheme

A plant scheme for carrying out the process according to the present invention is shown in **Figure 1**. The alcoholic solution of the lipid components is stored in the tank D-1, from which it is pumped into the supply line (1-2-3) of the alcohol solution by means of the peristaltic pump G-1 to the injector I-1 (nanoliposomes production area).

The aqueous solution of iron and ascorbic acid is stored in the tank D-2, from which it is pumped into the supply line (4-5-6) of the aqueous solution by means of the peristaltic pump G-2, which is also fed to the injector I-1.

The resulting hydroalcoholic nanoliposomes suspension (7) is then sent to the ultrasound device for homogenization/reduction of the average size, D-3, in which it is subjected to a sonication treatment with working cycles Z-1 (as described in Barba, AA, et al., *Ultrasonic energy in liposome production: process modeling and size calculation*, 2014. Soft Matter, 10, 2574-2581). The final homogenized nanoliposomes suspension is recovered through the line (8).

Parametric analysis: effect of the ratio of the aqueous solution flow rate to the alcohol solution flow rate

Figure 2 shows the average diameters (diagram A) and the polydispersity (diagram B) of the nanoliposomes as obtained in the injector I-1 (grey histograms), or after homogenization by sonication (histograms with 45° pattern), depending on the ratio of the flow rate of the aqueous solution to the flow rate of the alcoholic solution. The results are expressed as the average of three determinations and are reported with the relative standard deviation (the vertical bar).

It may be observed that an increase in the flow rate of the aqueous solution (and thus an increase of the flow rates ratio) causes a decrease in the average size of the nanoliposomes, both as such and after homogenization by sonication.

This effect is less clear as regards polydispersity, which increases with an increase in the ratio of the flow rates of non-sonicated nanoliposomes, while there is no evident effect of the flow rates ratio on the polydispersity index (PDI) of the nanoliposomes after homogenization by sonication .

Parametric analysis: effect of the expected concentration of lecithin in the hydroalcoholic solution after injection

Figure 3 shows the average diameters (diagram A) and the polydispersity (diagram B) of the nanoliposomes as obtained in the injector I-1 (grey histograms), or after homogenization by sonication (histograms with 45° pattern), depending on the concentration of phosphatidicoline (PC) expected in the hydroalcoholic solution after injection (line (7) in **Figure 1**). The results are expressed as the average of three determinations and are reported with the relative standard deviation (vertical bar).

It can be observed that an increasing in the lecithin concentration increases the average size of non-sonicated nanoliposomes, with a less monotonous effect for the nanoliposomes after homogenization by sonication. As far as the polydispersity is concerned, the effect is not monotonous and in any case very limited (PDI almost independent from PC concentration), both for non-sonicated nanoliposomes, and for nanoliposomes after homogenization by sonication.

Stability of nanoliposomes in distilled water over time

In order to ascertain the stability of the nanoliposomes upon storage, a preliminary test may consist of the observation the over time of the evolution of the iron contained in the nanoliposomes produced according to the invention and released in the dissolution medium (distilled water).

Therefore, **Figure 4** shows the results of these measures as a function of time, obtained over a period of 14 days. The empty symbols refer to the iron sulphate mass contained in the pellet, while the full symbols represent the iron released in deionized water.

As can be seen from the concerned diagram, the measurements confirm the substantial stability of the thus obtained nanoliposomes.

The present invention has been described with particular reference to some embodiments thereof but it should be understood that changes and modifications can be made by those skilled in the art without departing from the scope of the invention as described in the appended claims.

CLAIMS

1. A continuous or semi-continuous simil-microfluidic process for the preparation of nanoliposomes for the administration of iron and other micronutrients in nutraceutical or fortified food products, which process comprises the following operations:

a) producing a first alcoholic solution of phospholipids and cholesterol at a concentration of from 0.1 to 10 mg/mL;

b) producing a second aqueous solution containing iron(II) salts, such as ferrous sulphate, ferrous citrate and ferrous lactate, and possibly salts of other micronutrients selected from the group of metal elements and mixtures thereof, together with a reducing compound such as ascorbic acid or a salt thereof;

c) pumping said alcoholic and aqueous solutions through two volumetric pumps in flow rates of the order of mL/s and in a respective volume flow rate ratio of from 5:1 to 50:1;

d) contacting the flows consisting of said two solutions by making them pass through a device operating in simil-microfluidic regime, i.e. a device which effects formation of nanoliposomes by injection of said first solution stream into said second solution stream through a duct, for instance a hollow needle, of internal diameter below 1 mm, the duct of said second solution having an internal diameter of 2-5 mm and a length after the injection point of 150-200 mm;

e) subjecting to an ultrasound treatment the nanoliposomes suspension obtained from the previous operation, to reduce the size of the nanoliposomes produced and limit their polydispersity,

the nanoliposomes thus obtained having an average size not higher than 100 nm and a polydispersity index of less than 0.5.

2. A process according to claim 1, wherein the alcoholic solvent of said alcoholic solution is ethanol, and said iron(II) salts consist of ferrous sulphate.

3. A process according to claims 1 or 2, wherein said reducing compound is ascorbic acid or a salt thereof.

4. The process according to claim 3, wherein the weight ratio of iron to ascorbic acid is 1:6.

5. A process according to any one of claims 1 to 4, wherein said first alcoholic solution of phospholipids and cholesterol is at a concentration of 5 mg/mL.

6. A process according to any one of claims 1-5, wherein said phospholipids consist of phosphatidylcholine.

7. The process according to claim 6, wherein the ratio of phosphatidylcholine to cholesterol is equal to 2.5: 1.

8. A process according to any one of claims 1-7, wherein said alcoholic and aqueous solutions are fed to said device operating in a simil-microfluidic regime in a volume flow rate ratio equal to 10:1, and the ratio of the internal diameter of the duct of said first solution to the internal diameter of the duct of said second solution is equal to 1:5.

9. A process according to any one of claims 1-8, wherein the volume flow rate ratio of said aqueous solution to said alcohol solution is from 5 to 100.

10. The process according to claim 9, wherein said volume flow rate ratio of said aqueous solution to said alcohol solution is from 10 to 40.

11. A process according to any one of claims 1-10, wherein the polydispersity index of the obtained nanoliposomes is between 0.3 and 0.4.

12. Food products and nutraceuticals containing the nanoliposomes obtained with the process of claims 1-11.

13. Food products and nutraceuticals according to claim 12, for use in the administration of iron and other micronutrients in nutraceutical products or fortified food products.

14. Food products and nutraceuticals enriched with iron(II), wherein the enrichment is obtained using the nanoliposomes obtained with the process of claims 1-11 as a source of bioavailable iron.

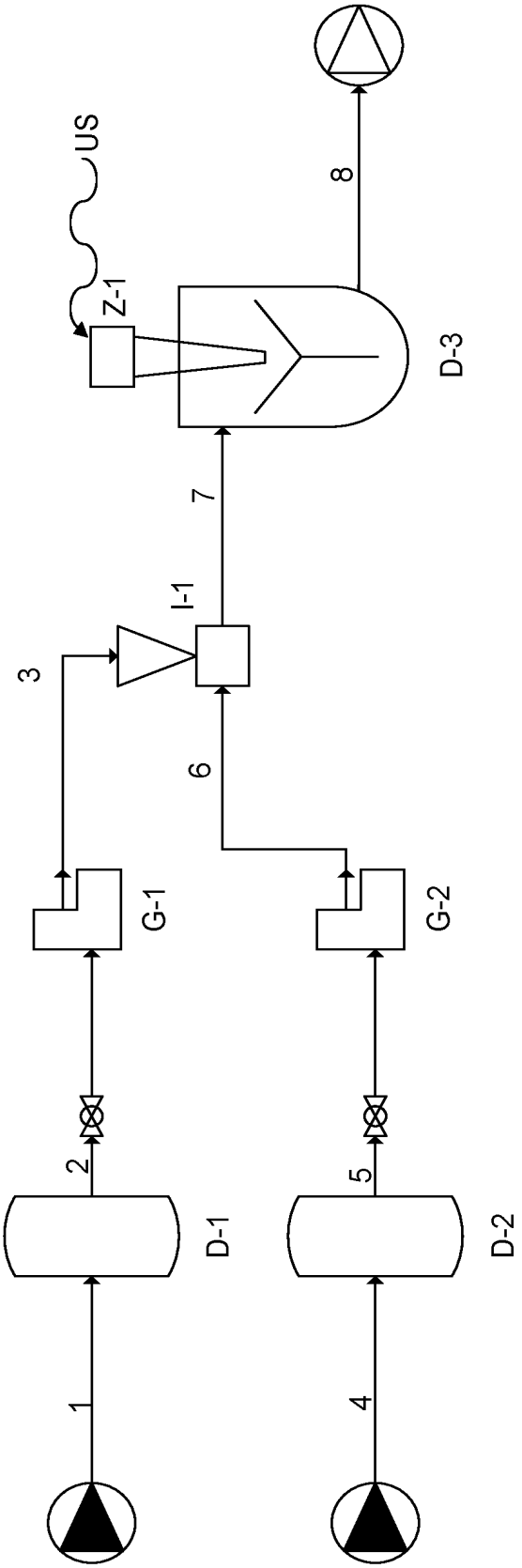

Fig. 1

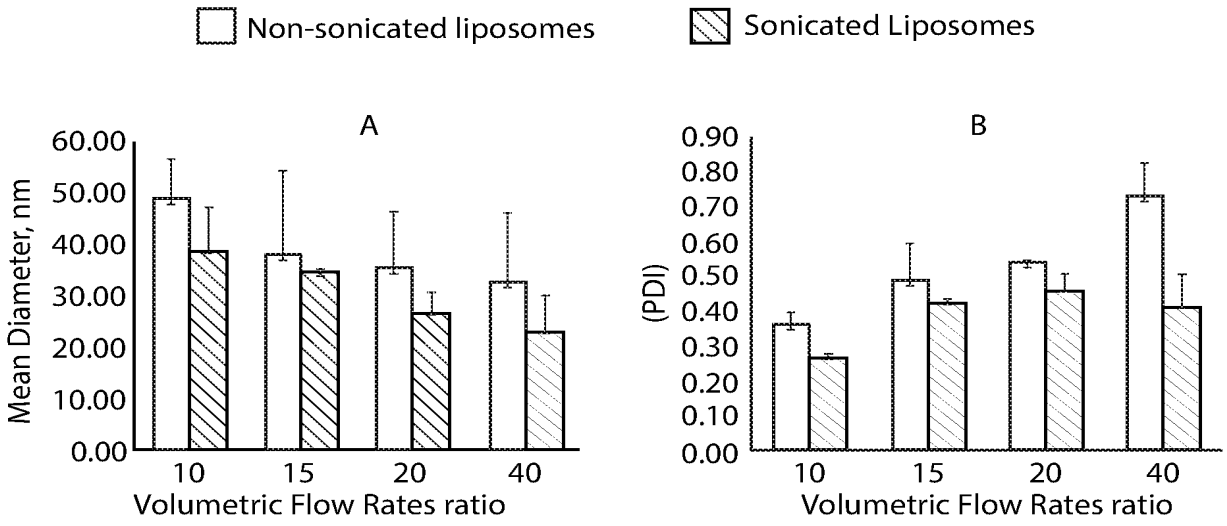
Non-sonicated liposomes
Sonicated Liposomes
A
Mean Diameter, nm
60.00
50.00
40.00
30.00
20.00
10.00
0.00
10
15
20
40
Volumetric Flow Rates ratio
B
(PDI)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
10
15
20
40
Volumetric Flow Rates ratio

Fig. 2

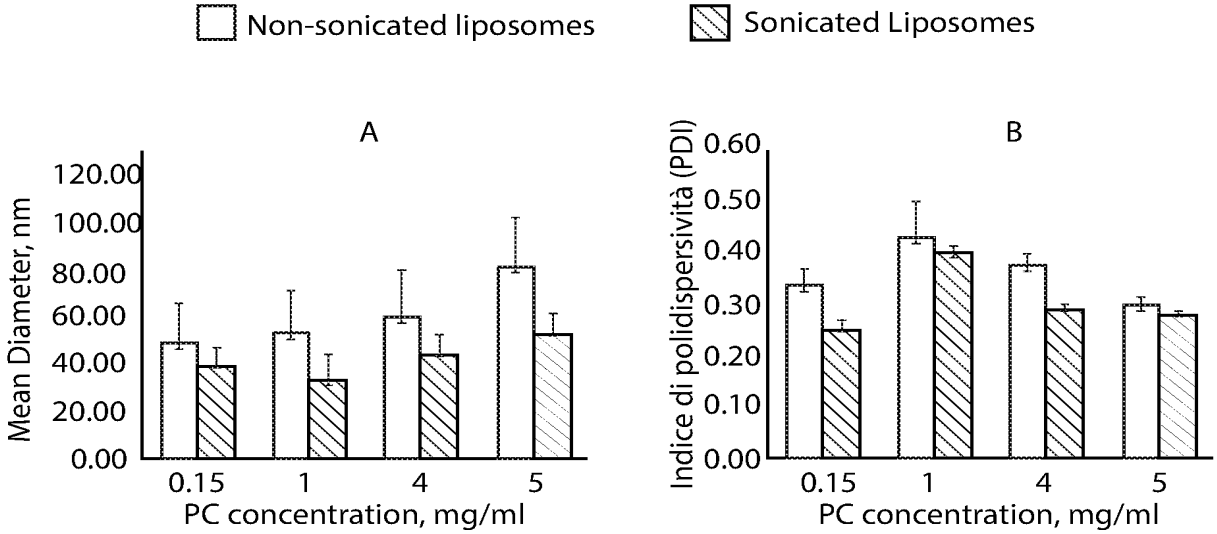
Non-sonicated liposomes
Sonicated Liposomes
A
Mean Diameter, nm
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0.15
1
4
5
PC concentration, mg/ml
B
Indice di polidispersività (PDI)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.15
1
4
5
PC concentration, mg/ml

Fig. 3

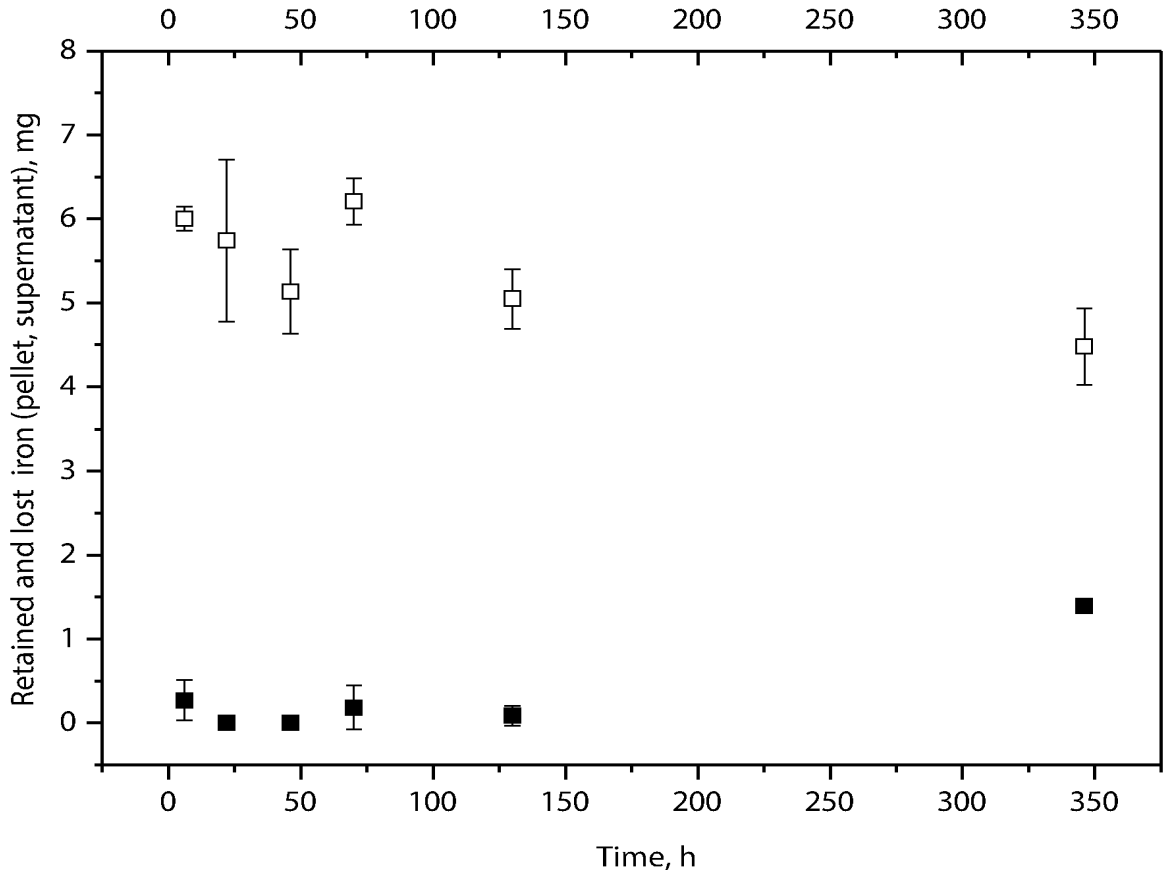
0
50
100
150
200
250
300
350
8
7
6
5
4
3
2
1
0
Retained and lost iron (pellet, supernatant), mg
Time, h

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2018/050165

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/127 A23L33/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2018	07/11/2018

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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