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Chapter 2

Phenomenological and Formulation Aspects in Tailored Nanoliposome Production

Sabrina Bochicchio, Gaetano Lamberti and Anna Angela Barba

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Abstract

Liposomes as cell-mimetic system have attracted wide attention of researchers in various branches of the drug delivery topic as they can be highly functionalized and personalized, thus solving the major drawbacks of bioactive molecules linked to their low stability, limited membrane permeability, short half-life and low bioavailability. The development of sustainable processes able to produce ad hoc liposomes in a rapid manner through the use of not-laboured techniques, avoiding drastic conditions, is of great relevance for the industrial sector. In this chapter, two novel liposome production processes, the ultrasound-assisted thin-film hydration and the simil-microfluidic techniques sharing the same size reduction/homogenization preparative step, are presented. The phenomenological aspects involved in vectors constitution through the duty cycle sonication process (bilayer rupture/vesicles formation mechanisms) and through the simil-microfluidic approach (intubated flows interdiffusion mechanisms) are described. Finally, two applications as case histories involving the use of the developed techniques for relevant classes of active molecule delivery are described. In particular, a pharmaceutical application concerns the encapsulation of short-interfering RNA (siRNA) molecule, used for gene therapy, inside cationic nanoliposomes, and a nutraceutical application consists in the production of ferrous sulphate anionic liposomal formulations with improved features compared to those already present on the market.

Keywords: ultrasonic size reduction, simil-microfluidic approach, nanoliposomes delivery systems, personalized carriers, cell-mimetic system, gene therapy, nutraceuticals

1. Introduction

Liposomes are closed vesicular structures, constituted by one or more phospholipid bilayers, which are formed when membrane lipids, such as phosphatidylcholine (PC) and

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cholesterol (CHO), are dispersed in an excess of water. Liposomes are efficient delivery systems, which are able to ensure a controlled and targeted release of active molecules due to their high biocompatibility, stability, biodegradability, intrinsic toxicity and immunogenicity [1]. Liposomes are also versatile systems in terms of dimensions and chemical modifications, they can be easily reduced in size and coated with different polymers and their surface can be chemically modified with specific ligands to give active targeting. These characteristics, together with their similarity to biological membranes, make them vectors of great interest when compared with other carriers. In particular, size and size distribution are key parameters determining liposomes performance as delivery systems. Compared with micrometre-sized carriers, produced by traditional microencapsulation techniques, nanoparticles have a larger interfacial surface area and have the potential to improve the solubility, enhance the bioavailability and improve the controlled release of the bioactive principle [2]. In nutraceutical applications, liposomes of nanoscale dimensions can improve taste, flavour, stability, absorption and bioavailability of the bioactive compounds [3–6]. From a pharmaceutical point of view, nanoparticles are preferred for their elongated retention time in the small intestine when compared to the larger structures [7] and are particularly desired due to the enhanced permeability and retention (EPR) effect [8]: liposomes with small dimensions can permeate through membrane fenestrations of diseased blood vessels penetrating into the tumour tissue [9].

Due to their favourable features, liposome-based products to be used in pharmaceutical and nutraceutical field have risen together with the need of large-scale and low-environmental impact techniques capable of producing significant amounts of liposomes in a short time and without the use of drastic conditions. Indeed, according to the Paris Agreement on climate change [10], innovation must go hand in hand with the developing of green products, improving the business processes and scaling up investments through greater energy and material efficiencies [11]. The energy efficiency and emissions reduction in the industrial sectors are the crucial point. All this constitutes the basis of the modern industrial manufacturing and the approach is referred as process intensification PI [12–14].

Nowadays, there are a wide set of possibilities to produce lipid-based drug delivery systems through the use of conventional or more recently discovered techniques [15–17]. The membrane contactor, the supercritical fluid and the microfluidic methods [18–20] are among the most recent.

However, despite the leaps and bounds made with the novel technologies in the last few years, the majority of these methods are characterized by high-energy request, long times of process, the use of toxic solvents together with a low productivity. In particular, the most used techniques, such as the ethanol injection [21] or the thin-film hydration (TFH) method, are bench-scale methods characterized by bulk discontinuous processes. Microfluidics is a relatively new technology used for the production of liposomes on nanometric scale [22]. The latter gives the possibility to produce, in a continuous manner, small unilamellar liposomes (SUVs) with a precise control on liposomes dimensional features by modulating the flows at

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micrometric scale; anyway the method is characterized by elevated costs of microfabrication and low-product volumes in output.

To overcome these limitations, in this work, two novel versatile and reliable techniques for nanoliposomes production, based on the use of ultrasound as process intensification tool, have been presented. At first, in order to produce, in a versatile manner, nanometric structures with the desired dimension, an ultrasound-assisted size reduction process was developed and coupled with the conventional thin-film hydration method. Subsequently, due to its reliability and versatility, the ultrasound-assisted process generated was also used for liposomes homogenization operation during vesicles production through a simil-microfluidic approach. In that regard, a semi-continuous apparatus, based on microfluidic principles, was expressly designed and fabricated in order to produce higher volumes of lipid vectors, potentially on production scale, directly of nanometric size, overcoming the limitations of the small output volumes typical of the conventional bench-scale techniques. The phenomenological aspects involved in vectors constitution were investigated and described for both the adopted setup. The two methods were finally adopted for short-interfering RNA (siRNA) and ferrous sulphate encapsulation in ad hoc-formulated nanoliposomes to be used in pharmaceutical and nutraceutical applications, respectively.

2. Novel developed techniques for liposomes production

2.1. Ultrasound-assisted thin-film hydration: layout, principles and phenomenological aspects

A versatile and reliable technique able to produce liposomes of different sizes to be used for disparate applications has been developed by coupling the conventional thin-film hydration method [16], which produces micrometric structures, with an ultrasound-assisted process developed to prepare, in a versatile manner, nanometric structures with the desired dimensions. In particular, the size of liposomes is determined during the production process, decreasing due to the addition of ultrasound energy. The energy is used to break the lipid bilayer into smaller pieces, then these pieces close themselves in spherical structures as phenomenologically detailed in Section 2.1.3.

2.1.1. Layout

Figure 1 shows a schematization of nanoliposomes production process through the ultrasound-assisted TFH technique developed. The setup is composed by four main sections: a feeding section where solutions are stored, an evaporation section, constituted by a rotary evaporator (Heidolph, Laborota 4002 Control), where solvents are removed, a production section composed of a tank where vesicles are formed and then homogenized through the use of an ultrasonic source (VCX 130 PB Ultrasonic Processors of Sonics & Materials Inc., CT, USA; maximum power of 130 W; frequency of 20 kHz), giving nanoliposomes as output product and, finally, a recovery section where vesicles are collected and characterized.

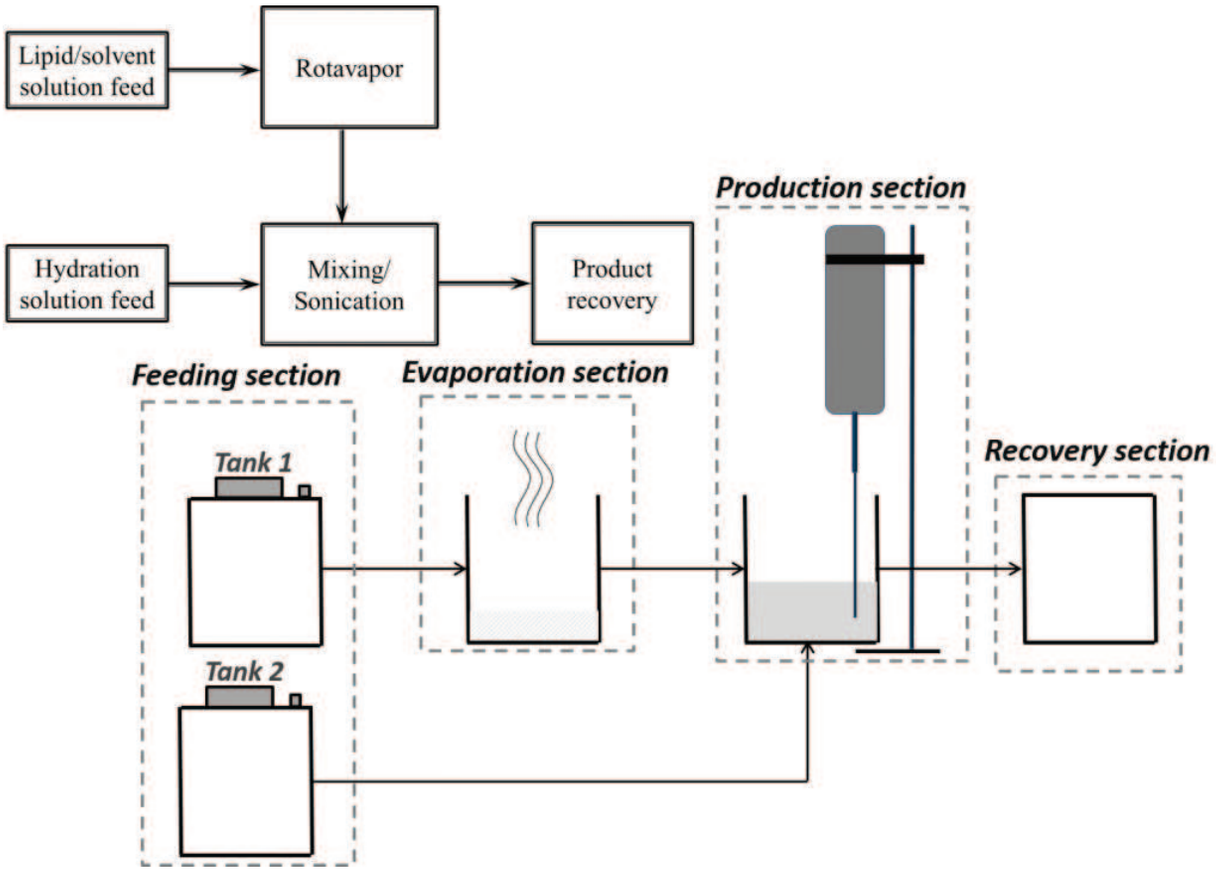

Figure 1. Nanoliposome production through the ultrasound-assisted thin-film hydration method; the main steps are reported: from Tank 1, the lipids/organic solvent solution (eventually containing the hydrophobic molecule to be encapsulated) is introduced in the evaporation section where, by means of a rotary evaporator, solvents are removed leading to the formation of a lipid film. This is then hydrated with a solution (eventually containing the hydrophilic molecule to be encapsulated) stored in Tank 2 of the feeding section. The suspension is homogenized through an ultrasound-assisted process leading to the nanoliposome formation. Finally, the suspension is recovered and characterized.

Briefly, for liposomes production at nanometric scale, at first lipids are dissolved in organic solvents (chloroform/methanol) eventually containing the hydrophobic drug to be encapsulated; after mixing, the solution is vacuum-dried. The dried lipid film, which is generated, is then hydrated at room temperature with water or other hydration solutions eventually containing the hydrophilic drug to be encapsulated and continuously stirred. A suspension containing multilamellar vesicles (MLVs) is produced, maintained at room temperature for several hours and then sonicated by applying a duty cycle purposely developed.

The duty cycle is a discontinuous process by which the liquid sample is sonicated in periodic time intervals followed by switch off in energy supply. It consists of few and short (in the order of seconds) irradiation rounds each followed by few and short pause in order to prevent thermal vesicle disruption, thus obtaining large vesicles (LVs). The sample is stored overnight at 4°C and protected from light in order to stabilize the produced LVs. Subsequently, in order to obtain SUVs, after the stabilization phase, the sample is sonicated again, up to more

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rounds. The process parameters influencing the sizing process are the ultrasonic amplitude (%), the power (W), the number of sonication cycles, the time of sample exposure to ultrasounds and the volume of the sample treated. As described in detail in Ref. [4] by using a 45% amplitude (percentage of maximum-deliverable power), treating 1-ml volume and by applying the duty cycle, starting from MLVs, after several irradiation rounds, LUVs with 1.416 ± 0.117-μm diameter size are obtained. Subsequently, after more sonication rounds, SUVs with a mean diameter size of 86 ± 33 nm are produced. Finally, after more irradiation rounds, SUVs of 51 ± 28 nm and 49 ± 26 nm are obtained, respectively. In **Figure 2**, produced vesicles from micro- to nanoscale are shown. The duty cycle sonication protocol, coupled with the traditional thin-film hydration method, gives several advantages over conventional liposome-sizing processes as detailed in the next paragraph; first of all, there is the possibility to change liposome dimensions according to the application requirements, avoiding the fixed pore size of the membranes used in the extrusion method.

2.1.2. Principles of the ultrasound process and benefits

Ultrasound is a mechanical vibration phenomenon having a frequency above the range of human hearing (>20 KHz). When ultrasound is adsorbed by a medium, acoustic vibrations increase kinetic energy, producing instantaneous temperature and pressure rise. The explanation of the process can be found in cavitation-wave hypothesis, proposed in 1960. According to this hypothesis, when pressure changes, there is a formation of cavities at liquid–gas interface. The collapse of these cavities generates shock-wave bubbles capable of resonance vibration and producing vigorous eddying or microstreaming. The stresses associated with the propagation of ultrasonic waves may be converted into thermal energy or into chemical energy. Ultrasound-assisted processes can be used for disparate industrial applications, that is, to homogenize, atomize, disperse, deagglomerate and sizing particles, emulsify, disintegrate cells and extracting protein or enzymes from them, to increase reaction speed, to clean and to degas liquids. For example, ultrasonic atomization takes advantage by ultrasound

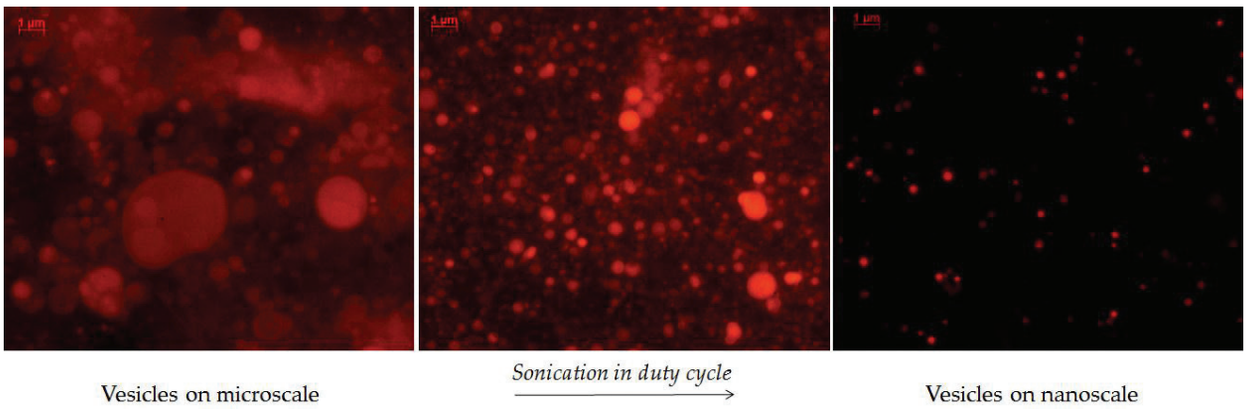

Figure 2. Fluorescence microscopy images of lipid vesicles labelled with Rhodamine B dye (100× objective). Right: Vesicles on microscale not subjected to the size reduction process. Left: Vesicles on nanoscale after sonication in duty cycle.

phenomenon to break up a liquid film into fine droplets. In this case, the phenomenology of the droplets formation process can be explained with cavitation-wave hypothesis or with the capillary-wave hypothesis [23] which analyse the behaviour of a liquid on the solid surface vibrating based on Taylor instability criteria [24]. When the vibration has a frequency of 30 Hz, above a threshold value of amplitude, on the liquid surface capillary waves are formed consisting in crests and troughs. At higher values of amplitude, liquid droplets, separated from the wave crest, are formed [25].

In general, ultrasonication is an easy and scalable process which shows competitive energy costs, applicable in a vast range of fields and for disparate applications. Regarding the particle size reduction, it takes advantage from ultrasound process of the ability to break up lipid structures. In case of nanoliposomes production, the energy is used to break the lipid bilayer into smaller pieces; then these pieces close themselves in spherical structures producing SUVs. In comparison with other sizing techniques, the ultrasonic, which once shows great potential due to reduced time spent and easiness in use, is a simple and efficient method to reduce liposome dimension [26, 27]. Ultrasonic process does not require a number of passages of liposome suspension through a membrane such as extrusion method, and high pressures are not required. Furthermore, the final dimensions of particles are not fixed to the pore size of the membrane, but it is possible to change the vesicle size according to the application requirements. This is possible by controlling the duty cycle, the discontinuous process before described in which the liquid sample is sonicated in periodic time intervals followed by switch off in energy supply. Finally, due to the critical importance to have a sterile environment during loaded SUVs production, another advantage is that sonotrode tip is simpler to clean and sterilize [4].

2.1.3. Phenomenological aspects

As described in Ref. [28], from a thermodynamics point of view, the free energy of the liposome membrane is mainly given by two contributions: the elastic energy due to membrane curvature and the tension energy due to the edge of the layer. Indeed, at the boundary of a bilayer, the polar heads have to be arranged as a semi-circle, in order to connect the two monolayers [29]. The elastic energy has been estimated by Helfrich [30] as proportional to the second power of twice the mean surface curvature, $1/R$ [m^{-1}], and to Gaussian surface curvature, $1/R^2$ [m^{-2}]; through the main elastic moduli, k_c [J] and $\overline{k}_c$ [J], the edge energy are assumed to be proportional to the length of the bilayer edge [30, 31], by the edge tension parameter, γ [J m^{-1}]. On the basis of simple geometrical reasoning [31], a disc initially of radius ρ_D [m], with a surface area $A_D = \pi\rho_D^2$, which is (partially) bended towards forming a sphere (equivalent in area, then with a sphere radius R_S [m] of $R_S = \rho_D/2$), has an edge length equal to $L = (2\pi\rho_D^2)\sqrt{1-(\rho_D^2/(4R^2)}$. Summarizing, for N vesicles with a mean curvature $1/R$, the total free energy could be estimated by Eq. (1) [1]

$$g = N\left(g_{\text{elastic}} + g_{\text{edge}}\right) = N\left\{\left[\frac{1}{2}\left(k_c + \frac{1}{2}\overline{k}_c\right)\frac{4\pi\rho_D^2}{R^2}\right] + \left[\gamma\left(2\pi\rho_D\right)\sqrt{1-\frac{\rho_D^2}{4R^2}}\right]\right\} \quad (1)$$

Therefore, the thermodynamic of the process could be described once the number and the size of the starting discs, N and ρ_D, as well as the curvature of the vesicle, $1/R$, are known;

after the estimation of the material parameters ($2k_c + \overline{k}_c$) and γ. In general, when the energy is supplied to the system, the curvature decreases (the radius of curvature increases), meaning that the spherical vesicles start to open, and the number of liposomes remains constant: for the first second of the process, the supplied energy is used just to increase the free energy of the membrane. During the following 9 s, the radius of curvature remains constant on a very high value because the membrane is flat, the radius of the equivalent disc starts to decrease and the number of structures increases. Both these phenomena are due to the fact that the membrane is being disrupted by the ultrasound energy, thus the total free energy increases, being stored in the structures as edge energy. The magnitude of the phenomena, which happen during this phase, is also dictated by the value of the power parameter $\dot{g}_0$ (the higher the power, the higher the number of discs produced and the smaller their size). During the following 20 s, the relaxation process starts, and the discs bend themselves towards the spherical configuration, since the total free energy in that configuration is lower (the elastic contribution is lower than the edge contribution). During this phase (the bending phase), the radius of curvature decreases (the curvature increases) and the total free energy decreases, no more entities were produced and their size remains constant (N and ρ_D are constants). Subsequently, the cycle starts again. During the first seconds, the discs open (R increases), the entities do not change the number and size (N and ρ_D remain constants) and the total free energy increases. During the remaining of supply-energy phase, the entities were flat discs, and then the energy was used to disrupt them: the curvature does not change, the number of entity increases and their size decreases. The total free energy still increases. Then, the relaxation phase takes place: the discs bend towards sphere, thus the radius of curvature decreases and the total free energy decreases too. The number and the size of entities remain constant.

2.2. Simil-microfluidic method: layout, principles and phenomenological aspects

A simil-microfluidic apparatus was expressly designed and fabricated in order to produce lipid vectors, potentially on production scale, directly on nanometric size, overcoming the limitations of the thin-film hydration technique (and other conventional production methods), which produces small output product volumes in a slow and discontinuous manner. Moreover, the method was developed to avoid the limitations related to the expensive devices needed and microfabrication costs of the microfluidic systems, by transposing their principles to a millimetre scale, drastically reducing the production costs and increasing the yields. With this aim, a new semi-continuous bench-scale apparatus was designed and developed and the ultrasonic energy was used again as an intensification tool for liposome homogenization. The protocol based on the simil-microfluidic approach basically consists in the realization of a contact between two flows, lipids/ethanol and water solutions, inside a millimetric tubular device where interdiffusion phenomena allow the formation of lipid vesicles as detailed in the subsequent paragraphs.

2.2.1. Layout

Figure 3 shows a schematization of the developed simil-microfluidic apparatus. The setup is constituted by five main process sections: a feeding section, a pumping section, a production section, a homogenization section and a recovery section.

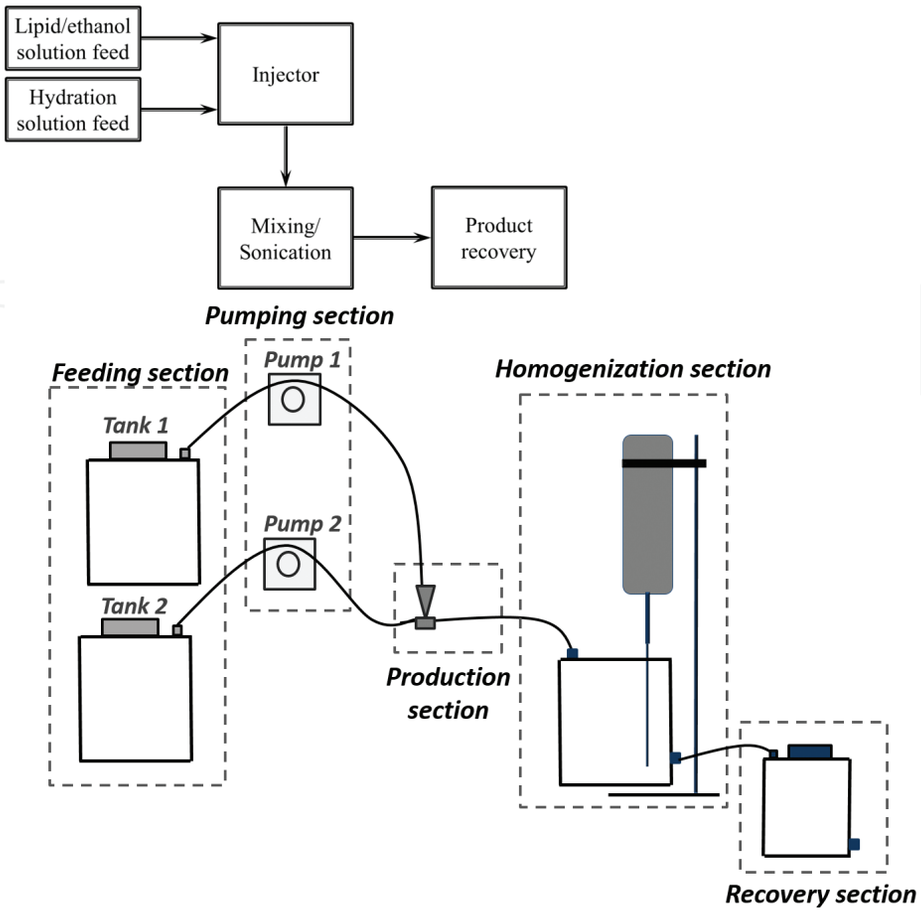

Figure 3. Nanoliposome production through the simil-microfluidic setup. The main sections are reported: feeding, pumping, production, homogenization and recovery. From Tanks [1–2], lipids/ethanol (eventually containing the hydrophobic molecule to be encapsulated) and water (eventually containing the hydrophilic molecule to be encapsulated) solutions are pushed, through peristaltic pumps (Pumps 1–2), to the production section. Here, after the lipid solution injection into the polar phase, nanometric vesicles are formed. The hydroalcoholic solution is then recovered and homogenized through an ultrasound-assisted process. Finally, the suspension is recovered and characterized.

In particular, the feeding section consists in two lines. The first one is made up by a stirred tank filled with lipids/ethanol solution (Tank 1 of **Figure 3**), in which a hydrophobic active molecule to be encapsulated, conveyed in a silicon tube with an internal diameter of about 2 mm, is eventually dissolved. The second line includes a stirred tank filled with the hydration solution (Tank 2 of **Figure 3**), which can be pure water or an aqueous solution, eventually containing the hydrophilic active molecule to be encapsulated, conveyed in a flexible silicone tube with an internal diameter of few millimetres.

The pumping section is composed by two single-head peristaltic pumps (Verderflex OEM mod. Au EZ) indicated as Pumps 1–2 in **Figure 3**.

The lipids/ethanol solution tube ends with several tenths of millimetres internal diameter needle inserted into a silicon tube, an extension of the water tube. This is the production section sketched in **Figure 3**. In this section, an interdiffusion of the two pushed liquids occurs leading to the formation of liposomes on nanometric scale; the suspension is then collected inside a tank and subjected to a homogenization in order to optimize vesicles size distribution. The suspension is finally recovered and characterized.

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Briefly, the process starts when lipids/ethanol and water solutions are pushed through peristaltic pumps into the production section, where liposomal vesicles are formed directly on nanometric size (the phenomenology behind vesicles formation through a simil-microfluidic approach will be discussed in the next paragraph). The formed hydroalcoholic solution is recovered and subjected to a homogenization process through the duty cycle sonication protocol, previously described for the ultrasound-assisted thin-film hydration method [28] (Section 2.1.1).

2.2.2. Phenomenological aspects

From a phenomenological point of view, liposome formation is governed by the molecular diffusion between two phases: the organic solvent, in which the lipids are solubilized, and the water; the latter simultaneously diffuses into the organic solvent in order to reduce its concentration below the critical value required for the lipid's solubilization. During the diffusion process, lipid vesicles on nanometric scale start to form through a mechanism called 'self-assembly', according to the theory by Lasic and Papahadjopoulos [32]: lipids dissolved in an organic solvent are in the form of bilayer fragments (phospholipid bilayer fragments, BPFs), the interdiffusion of the water and the organic solvent reduces the solubility of the lipids in the solvent causing thermodynamic instability of BPF edges, inducing the curvature and the closure of bilayer fragments which allow the formation of liposomal vesicles [32]. In the simil-microfluidic setup developed, through the use of constructive expedients (millimetric tubes, peristaltic pumps and injection needle), the reproduction of the laminar flow regime was possible, all the Hagen-Poiseuille assumptions being satisfied, that is, the Reynolds number was found to be less than 2100 for all the volumetric flow rate conditions tested; the piping length in which the two phases interdiffuse was longer than the 'entrance length' required to obtain the parabolic profile [33]. In particular, for a microfluidic system and thus for a laminar flow, liposome formation occurs at the interfaces between the alcoholic and water phases, when they start to interdiffuse in a direction normal to the liquid flow stream. Changes in flow conditions result in size variations of the insertion section of the organic phase reflecting on the vesicles dimensional features. In particular, increasing the volumetric flow rates ratio, the size of the insertion section of the organic phase decreases; this leads to a major dilution of the organic phase limiting the formation of long BPFs, thus inducing the production of liposomal structures of small dimensions. In general, it was shown that the variation in shear forces at the interface of the two fluids has no consequence on liposome structure. In particular, maintaining constant the volumetric flow rates ratio and changing both the buffer and the lipid alcoholic solutions volumetric flow rates, Jahn and collaborators have demonstrated that it is not the magnitude of the shear forces between the parallel-layered stream in having significant impact on liposome's size and size distribution but the stream width (which depends on the volumetric flow rates ratio) [20]. Due to the developed apparatus, the phenomenology connected to the vesicles formation through a microfluidic approach was achieved, exceeding the limit of the bulk methods where a driving force of entropic nature leads to the liposome formation. Local fluctuations of the lipid concentration in a bulk solution make difficult to control the size and the polydispersity of the produced vesicles. On the contrary, the presence of intubated laminar flow with the relative matter diffusive transport allows to minimize the fluctuations of the lipid concentration inside the tubes and to modulate the size and the size distribution of the final vesicles.

2.2.3. Influence of process parameters on liposome dimensional features

When using the simil-microfluidic approach, the lipid concentration and the volumetric flow rate ratio [Vhs/Vls] have a great influence on liposome's size distribution as described in Ref. [34] and also found by Jahn and collaborators for a microfluidic hydrodynamic-focusing platform [35]. In particular, increasing the ratio between the water volumetric flow rate to the lipids-ethanol volumetric flow rate, the PDI value increases as shown in **Figure 4B**. On the contrary, the effect of the ultrasound-assisted process in reducing PDI and thus in ameliorating their size distribution (homogenizing) can be observed (**Figure 4B**).

Another crucial parameter affecting nanoliposome's dimensional features is the lipid concentration. In particular, as visible in **Figure 5A**, increasing lipid concentration, the liposome diameter also increases. This can be explained by the fact that at equal fluid dynamic conditions, a greater number of lipids impact at the same alcohol/water interface area dissolving in the same water volume, thus joining to form larger vesicles. The sample seems to be better homogenized at the higher-tested lipid concentration (**Figure 5B**).

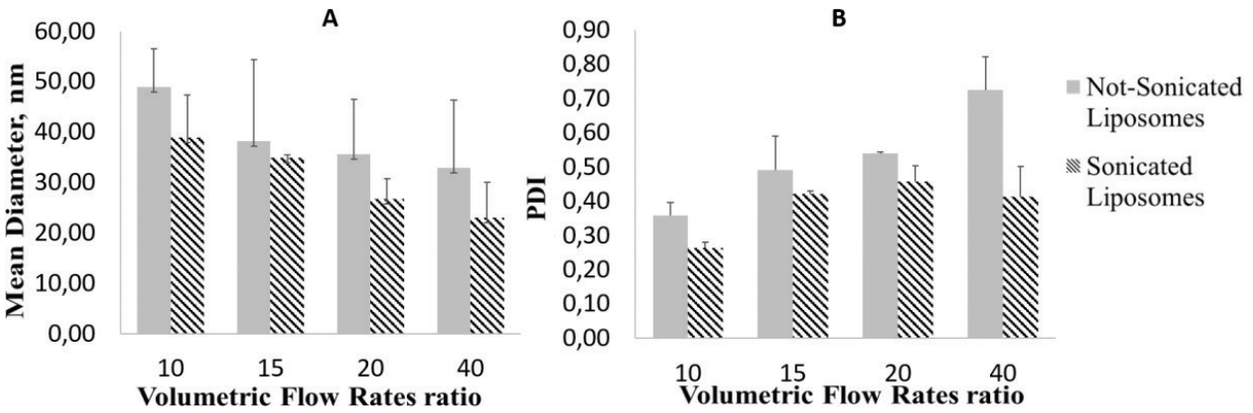

Figure 4. Liposome diameter size (A) and polydispersity index (PDI) (B) before and after sonication treatments at different volumetric flow rates [34].

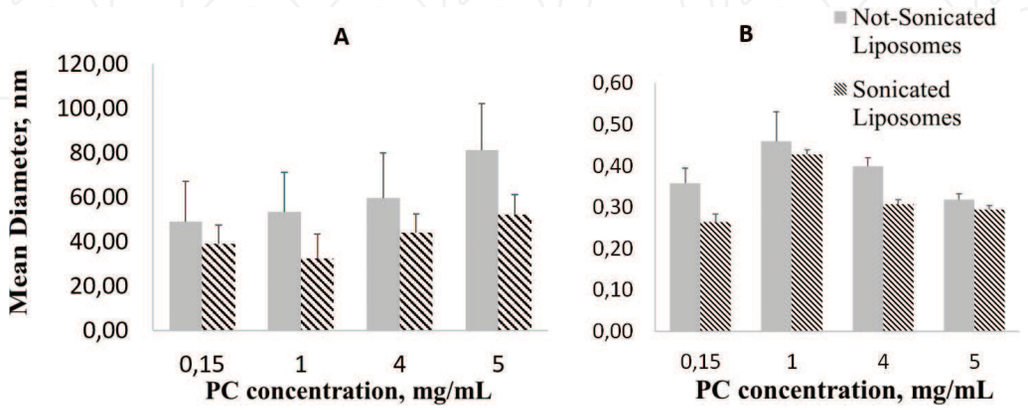

Figure 5. Liposome diameter size (A) and polydispersity index (PDI) (B) before and after sonication treatments at different phosphatidylcholine (PC) concentrations in the hydroalcoholic solution [34].

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3. Case histories

3.1. Pharmaceutical application: nanoliposome vectors for siRNAs delivery

The short-interfering RNAs are double-stranded RNA molecules able to target disease components, at genetic level that are considered 'undruggable' with the conventional medicines; thus, their use in the development of innovative gene therapies is growing faster in recent years. Due to their low stability in physiological fluids, low-membrane permeability and their short half-life in the circulatory system, siRNAs are not useable in their naked form and require to be encapsulated in suitable carriers.

As described in Ref. [36], siRNA sequences directed against E2F1 transcription factor (siE2F1-1324) were encapsulated inside positively charged vesicles purposely designed and produced to enhance the interaction with both the negatively charged siRNA, improving its encapsulation efficiency, and the cell-plasmatic membrane, also negatively charged, improving siRNA incorporation in the target cells [37, 38]. It was demonstrated that E2F1 promotes the aggressiveness of human colorectal cancer by activating the ribonucleotide reductase small subunit M2 whose high expression induces cancer and contributes to tumour growth and invasion [39]. Due to the observed correlation, the inhibition of E2F1 expression was studied as a potential way to treat colorectal cancer by encapsulating siE2F1 in cationic nanoliposomes suitably produced by the ultrasound-assisted technique developed. The loaded nanoliposomes were then transfected in human cell lines and in intestinal human biopsy fragments (collected from IBD donors during lower endoscopy performed for colonic cancer screening) to investigate their in vitro and ex vivo silencing activity. In particular, siE2F1 nanoliposomes were transfected in HT29 human colon adenocarcinoma cell line, where conditions are more reproducible, and in cultured human biopsies, in which the cell-cell interactions, thus the human intestinal mucosa cytoarchitecture, are preserved unlike isolated cell cultures.

3.1.1. Materials and methods

Formulation: Nanoliposomes loaded with siRNA sequences for E2F1 expression inhibition were designed and produced by using cholesterol (CAS 57-88-5), L-α-phosphatidylcholine from egg yolk (CAS 8002-43-5) and dioleoyloxy propyl-N,N,N-trimethylammonium propane (DOTAP) (CAS 132172-63-1, >99 % pure), purchased from Sigma-Aldrich (Milan, Italy). In designing the liposome bilayer, the cationic DOTAP phospholipid was chosen to electrostatically interact with the negative siRNA molecules, promoting siRNA encapsulation inside the lipid vesicles, and to interact with the negative cell membrane. The charge ratio between DOTAP and siRNA sequences used was 8.5:1 (±), selected on the basis of previous work [40].

The siRNA sequence direct against E2F1, the siE2F1-1324, was selected [41, 42]. siE2F1-SUV complexes were produced using the thin-film hydration method [16] followed by duty cycle sonication [28]. Briefly, PC, DOTAP and CHOL at 3:0.3:1 (mol:mol) ratio were dissolved in chloroform/methanol at 2:1 (vol/vol). The solvent was removed and the produced lipid film was hydrated with a phosphate buffer solution (PBS; potassium phosphate monobasic of 0.2 M,

sodium hydroxide of 0.2 M, pH 7.4) containing siE2F1-1324 at 8 μM. siRNA-positive and -negative controls (scramble siRNA) were also encapsulated. Finally, the above-described steps were followed also for unloaded liposome production with the only difference in the hydration solution which was pure PBS not containing siRNA sequences. By this way, multilamellar vesicles were achieved, maintained at room temperature for 2 h and then diluted obtaining an siRNA concentration of 4 μM for the loaded samples with a 1:260 (w/w) siRNA/total lipid ratio.

Production: In order to obtain nanoliposomes, samples were subjected to the duty cycle sonication process through the developed ultrasound-based size reduction method and previously described (Section 2.1.1).

Unloaded and siRNA-loaded SUVs were morphologically characterized through an optical microscope, equipped with software to capture the images in the fluorescence field (Axioplan 2- Image Zeiss, Jena, Germany). The size and zeta-potential determinations were performed by using the ZetasizerNano ZS (Malvern, UK) with non-invasive backscatter (NIBS) optics. The resulting particle size distribution was plotted as the number of liposomes versus size. The encapsulation efficiency (E.E.) by spectrophotometric (Lambda 25 UV/VIS Spectrophotometer, λ = 260 nm for RNA molecules) and electrophoretic assay (run on 1.5% agarose gel) was evaluated. The E.E. was determined as the percentage of siRNA encapsulated into SUVs, calculated subtracting the amount of siRNA present in the supernatant of the centrifuged sample from the total amount of siRNA included in the formulation, to the initial amount of siRNA used.

In order to evaluate siRNA-nanoliposome cytotoxicity and their potential in E2F1 silencing, the complexes were transfected at 200 nM in human colorectal adenocarcinoma cell lines HT29 and in human colon mucosa biopsies, isolated from donors during colonoscopy.

3.1.2. Results and discussion

The production process (formulation and manufacturing) is of crucial importance in preparing siRNA-liposome complexes with the desired shape and size without damaging siRNA's integrity. The shape of liposomes is the main factor affecting carrier's entry in the cellular compartment. Spherical nanoparticle's uptake in mammalian cells was demonstrated to be 3.75–5 times more than rod-shape nanoparticles, indicating that the carrier's curvature can affect the entry in the plasma membrane [43]. In that regard, spherical SUVs were obtained through the ultrasound-assisted method adopted which has been successfully used to produce stable siRNA-SUV complexes on nanometric scale. The achieved siRNA-liposomes, with a mean diameter size of 38 nm (**Table 1**), are useful for the EPR effect, which involves carrier's extravasation through tumour vascular fenestrations of 50–100-nm range size. Another important feature is the surface charge of liposomes. Zeta-potential (ζ) was investigated for both unloaded and SUVs encapsulating siRNA samples; the results are presented in **Table 1**. The positive zeta-potential makes the produced liposomes applicable for the encapsulation of negative siRNA molecules and also promotes the fusion with the negatively charged cell membrane. The ζ-value of the unloaded liposomes (27.90 ± 1.60) appears to be significantly higher than the zeta-potential of the siE2F1-1324-SUV sample (18.02 ± 1.07 mV) suggesting that a strong complexation in addition to the siRNA core encapsulation occurred for the loaded structures.

Properties	siE2F1-1324	Unloaded SUVs
SUVs size [nm] ± SD	38.1 ± 5.6	24.9 ± 5.8
PDI ± SD	0.4 ± 0.02	0.26 ± 0.005
Zeta-potential [mV] ± SD	18.0 ± 1.07	27.9 ± 1.60
Encapsulation efficiency [%]	100	–

Encapsulation efficiency (E.E.) for siRNA-loaded nanoliposomes. Results are expressed as average of three determinations with SD as standard deviation [36].

Table 1. Size, PDI and zeta-potential of unloaded and siE2F1-1324-loaded small unilamellar vesicles (SUVs) produced.

Considering the high degradability of siRNA molecules, thus the difficulty in preserving them during all production steps, one of the main goals was to produce liposomes on nanometric size ensuring the integrity of siRNA's molecular structure at the end of the process as well as preserving their biological activity. siRNA sequences were encapsulated with a 100% E.E. showing the efficacy of both the production technique and the formulation adopted. Moreover, electrophoretic studies have showed the high complex stability, which is a very important parameter to take into account due to the high degradability and toxicity of free siRNA molecules. This result indicates the safety of the developed ultrasound-assisted technique which allows preserving siRNA integrity since no evidence of nucleic acids degradation was visible through the electrophoretic assay.

Regarding the unspecific toxicity of SUVs, results have indicated that siRNA-nanoliposome complexes are far less toxic than Lipofectamine®2000, a commonly used transfection agent which was also investigated in order to have a comparison with the developed liposomal vesicles. siE2F1-SUVs were also able to significantly reduce the vitality of the HT29 colon carcinoma cells, thus proving the effectiveness of the complexes and their ability in siE2F1 delivery, which finally down-regulates cell growth. SUVs were able to enter the cell and release siE2F1 without any toxic effects. Finally, a successful uptake and an E2F1-silencing effect were also observed in cultured human colon mucosa biopsy, achieving an E2F1 protein inhibition till 80.5%, with a patient-dependent response. It can be stated that the size reduction process through sonication in duty cycle is a far less complex and more rapid method for liposome size reduction than the one usually adopted and is able to produce liposomes in the nanometric size range (which can thus take advantage of the EPR effect) with high degree of size homogeneity and 100% encapsulation efficiency, relevant feature that can guarantee a uniform behaviour in terms of delivery properties [36].

3.2. Nutraceutical application: nanoliposome vectors for ferrous sulphate delivery

Anaemia, caused by iron deficiencies, is one of the most widespread nutritional deficiencies, affecting globally two billion people [44]. Despite the success of iron food fortification, particularly in developing countries, the lack of a robust, simple and easy-to-transfer fortification technology has limited this technology [45]. Moreover, the supplementary micronutrient products present on market in the form of tablet or capsules have to be improved in quality and variety in order to increase their availability and access in the commercial

sector [46]. In order to meliorate the supplementary iron products currently on the market, often composed by micrometric particles, sometimes containing the less absorbable ferric form of iron and obtained, in the most of the cases, by using ineffective production processes and drastic conditions, ferrous sulphate nanoliposomes were produced by using the developed simil-microfluidic apparatus.

3.2.1. Materials and methods

Formulation: L-α-phosphatidylcholine from soybean, Type II-S, 14–23% choline basis (CAS n. 8002-43-5), cholesterol (CAS n. 57-88-5), ferrous sulphate heptahydrate (CAS n. 7782-63-0), ascorbic acid (CAS n. 50-81-7) and ethanol of analytical grade (CAS n. 64-17-5) were purchased from Sigma-Aldrich (Milan, Italy) and used for liposome production. Unloaded and ferrous sulphate-loaded nanoliposomes were produced by using the simil-microfluidic bench-scale apparatus developed whose layout and main process steps are described in Section 2.2.1. In particular, a 10:1 (Vhs/Vls) volumetric flow rate ratio and a 5-mg/ml lipid concentration in the final hydroalcoholic solution were used for liposome preparation.

Production: Briefly, a lipid/ethanol solution was obtained by dissolving PC and cholesterol in 10 ml of ethanol. Cholesterol was used at 2.5:1 (mol/mol) PC/CHOL ratio which corresponds to the typical composition of the cell membrane, as suggested by Abbasi and Azari [47] and was added to the formulation in order to stabilize the loaded vesicles. Ferrous sulphate heptahydrate and ascorbic acid were dissolved in 100 ml of deionized water, which was used as hydration solution. Ascorbic acid was added as an anti-oxidant to preserve the ferrous ion against oxidation in a ferrous/ascorbic acid. It has been shown that the co-addition of ascorbic acid and iron in a 2:1 molar ratio (6:1 weight ratio) increases iron absorption from foods twofold to threefold in adults and children [48–50]. In particular, different formulations were produced which differ from each other for the ferrous sulphate/total formulation components (lipids, ascorbic acids and ferrous sulphate) weight ratio (w/w) used. Starting from a 0.06 ferrous sulphate/total components weight ratio, selected from Xia and Xu [51], nanoliposomes were also produced by using a 0.02 ferrous sulphate/total components (w/w) ratio and maintaining all the other chemical and adopted process parameters constant. In order to have a comparison with the ferrous sulphate-loaded particles, unloaded nanoliposomes were produced by adoperating the same formulation and process conditions but using, as hydration solution, pure deionized water without the addition of ascorbic acid and iron.

Unloaded and ferrous sulphate-loaded SUVs were at first characterized in terms of morphology, size and zeta-potential (ZetasizerNano ZS, Malvern, UK). The resulting particle size distribution was plotted as the number of liposomes versus size.

The encapsulation efficiency was determined as the percentage of ferrous sulphate encapsulated in nanoliposomes to the initial amount of ferrous sulphate included in the formulation. Triton X100 at 1% (v/v) was used in order to lyse the nanoliposomes and analyse the encapsulated ferrous sulphate. Iron determination was performed by the 1,10-phenanthroline colorimetric method through UV spectrophotometric assay (Lambda 25 UV/VIS Spectrophotometer, Perkin Elmer, Monza, Italy). A λ = 510 nm, typical of the 1,10-phenanthroline-Fe^{2+} ions complex, was considered.

3.2.2. Results and discussion

The simil-microfluidic bench-scale apparatus developed has allowed to successfully produce iron-loaded nanoliposomes in the desired range size. The method has permitted to produce ferrous sulphate-nanostructured vectors without the use of drastic conditions, such as solvents and/or high pressure, currently used in literature and also at industrial scale for iron particle manufacturing by using discontinuous and laborious processes such as reverse phase evaporation, thin-film hydration and homogenization freeze-thawing production methods [47, 51–53]. A part of the drastic conditions used, limits in the output volumes of final product, usually ranging from 10 to 60 mL with the above-mentioned techniques, represents another crucial problems directly linked with high commercial costs of supplemental products which, for this reason, are not yet widely used as very proper therapies. With the simil-microfluidic setup developed, by using the ultrasound as tool for the process intensification, it was possible to obtain a massive output with the minimum energy, costs and time by operating in a semi-continuous manner. In particular, spherical liposomes were obtained in a nanometric range size as shown in **Table 2**.

Taking into account that the iron solubility is very dependent on the size and the shape of the iron particle complexes, characteristics which are governed by the manufacturing process [45], the simil-microfluidic setup realized was successfully applied for ferrous sulphate nanoliposomes production. In that regard, particles on nanometric scale are required to maintain the transparency of clear beverages during enrichment: carriers have to be small enough so as not to scatter light and be detected by naked eye [54].

The nanoscale plays a crucial role also for other forms of iron supplementation such as oral formulations, the first choice to replace normal iron levels. In this case, the size of nanoparticle systems has a remarkable influence on carrier's uptake after their administration: in many works, it has been proven that nanostructured delivery systems yield an increase in drug uptake, enhancing the intestinal absorption of the active principle [55, 56]. As shown in **Table 2**, vesicles of 48–65-nm diameter range size have been successfully obtained through the developed setup with PDI values of 0.38 ′ 0.01 and 0.63 ′ 0.12, respectively, for the 0.06 and 0.02 (w/w) formulations produced. Due to the presence of polyunsaturated fatty acids (linoleic and oleic acids) composing the phosphatidylcholine, vesicles presented negative zeta-potential values (more negative for the 0.06, w/w formulation due to the presence of large amounts of ascorbic acid which decreases the pH of the sample) and an encapsulation efficiency that increases with

Properties	0.06 (w/w)	0.02 (w/w)
SUVs size [nm] ± SD	47.80 ± 6.46	65.16 ± 15.48
PDI ± SD	0.38 ± 0.01	0.63 ± 0.12
Zeta-potential [mV] ± SD	−41.05 ± 0.7	−19 ± 0.55
Encapsulation efficiency [%]	22.33 ± 0.58	52.2 ± 1.41

Results are expressed as average of three determinations with SD as standard deviation.

Table 2. Size, PDI, zeta-potential and encapsulation efficiency of loaded small unilamellar vesicles (SUVs) produced, obtained at different weight ratios of ferrous sulphate to the total formulation components.

increasing of the lipid amount with respect to those of iron. In particular, as can be seen in **Table 2**, the encapsulation efficiency increases from about 22 to 52% when the weight ratio between ferrous sulphate and the total lipid decreases from 0.06 to 0.02 (w/w).

4. Conclusions

Based on the use of ultrasound as alternative energy resource, a solid particles size reduction/homogenization process was developed and coupled with the bench-scale conventional thin-film hydration method. The technique was developed in order to produce, in a versatile manner, nanometric structures, with the desired dimension.

Moreover, due to its easiness, reliability, versatility and its great potential in reducing time spent, the ultrasound intensification tool was also used for liposome homogenization operation during vesicles production through the developed simil-microfluidic technique.

The phenomenology involved in liposome formation was described for both the methods; applications regarding the entrapment of active molecules were also described as case histories.

siRNA-nanoliposome complexes (for gene therapy application) were produced for the inhibition of E2F1 protein expression, studied as a potential way to treat colorectal cancer. By the ultrasound-assisted thin-film hydration technique, nanoliposomes with 33–38-nm range size and 100% siRNA encapsulation efficiency were obtained. The produced loaded SUVs demonstrated a very low cytotoxicity in cells when compared with the commercial transfection agent Lipofectamine®2000 and an excellent uptake in the cultured human colon mucosa tissues. A remarkable effect on anti-E2F1 expression after a transfection of siE2F1-1324-SUV sample has been demonstrated also in a dynamic human model such the colon tissue microenvironment.

For nutraceutical application, nanoliposomes loaded with ferrous sulphate with good dimensional features (48–65 nm vesicles) and encapsulation efficiency were successfully produced using the developed simil-microfluidic apparatus, avoiding the use of toxic solvents and drastic conditions.

All the achieved positive results endorse the usefulness of both the formulative and the plant-engineering approaches adopted for nanostructured vectors production to be used in pharmaceutical and nutraceutical applications.

Author details

Sabrina Bochicchio[1,2], Gaetano Lamberti[1] and Anna Angela Barba[2]*

*Address all correspondence to: aabarba@unisa.it

1 Department of Industrial Engineering, University of Salerno, Fisciano, Salerno, Italy

2 Department of Pharmacy, University of Salerno, Fisciano, Salerno, Italy

References

[1] Sawant RR, Torchilin VP. Challenges in development of targeted liposomal therapeutics. The AAPS Journal. 2012;**14**(2):303-15

[2] Singh H. Nanotechnology applications in functional foods; opportunities and challenges. Preventive Nutrition and Food Science. 2016;**21**(1):1

[3] Reza Mozafari M, Johnson C, Hatziantoniou S, Demetzos C. Nanoliposomes and their applications in food nanotechnology. Journal of Liposome Research. 2008;**18**(4):309-27

[4] Bochicchio S, Barba AA, Grassi G, Lamberti G. Vitamin delivery: carriers based on nanoliposomes produced via ultrasonic irradiation. LWT-Food Science and Technology. 2016;**69**:9-16

[5] Srinivas PR, Philbert M, Vu TQ, Huang Q, Kokini JL, Saos E, et al. Nanotechnology research: applications in nutritional sciences. The Journal of Nutrition. 2010;**140**(1):119-24

[6] Putheti S. Application of nanotechnology in food nutraceuticals and Pharmaceuticals. Journal of Science and Technology. 2015;**2**(10):17-23

[7] Huang Q. Nanotechnology in the Food, Beverage and Nutraceutical Industries: Elsevier; 2012, Sawston, Cambrige, UK

[8] Bregoli L, Movia D, Gavigan-Imedio JD, Lysaght J, Reynolds J, Prina-Mello A. Nanomedicine applied to translational oncology: a future perspective on cancer treatment. Nanomedicine: Nanotechnology, Biology and Medicine. 2016;**12**(1):81-103

[9] Kibria G, Hatakeyama H, Sato Y, Harashima H. Anti-tumor effect via passive anti-angiogenesis of PEGylated liposomes encapsulating doxorubicin in drug resistant tumors. International Journal of Pharmaceutics. 2016;**509**(1-2):178-87

[10] Abeysinghe A, Barakat S. The Paris Agreement. Options for an effective compliance and implementation mechanism, 2016, http://pubs.iied.org/pdfs/10166IIED.pdf

[11] Burck J, Bals C, Rossow V. The Climate Change Performance Index: Results 2015: Germanwatch, Berlin; 2014

[12] Dalmoro A, Barba AA, Lamberti G, d'Amore M. Intensifying the microencapsulation process: Ultrasonic atomization as an innovative approach European Journal of Pharmaceutics and Biopharmaceutics. 2012;**80**(3):471-7

[13] Van Gerven T, Stankiewicz A. Structure, energy, synergy, time the fundamentals of process intensification, Industrial & Engineering Chemistry Research; 2009;**48**:2465-74

[14] Charpentier JC. In the frame of globalization and sustainability, process intensification, a path to the future of chemical and process engineering (molecules into money). Chemical Engineering Journal 2007;**134**:84-92

[15] Meure LA, Foster NR, Dehghani F. Conventional and dense gas techniques for the production of liposomes: a review. AAPS Pharmscitech. 2008;**9**(3):798-809

[16] Bangham A, Horne R. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. Journal of Molecular Biology. 1964;**8**(5):660-IN10

[17] Wagner A, Vorauer-Uhl K. Liposome technology for industrial purposes. Journal of Drug Delivery. 2010;**2011**

[18] Jaafar-Maalej, Chiraz, Catherine Charcosset, and Hatem Fessi. A new method for liposome preparation using a membrane contactor. Journal of Liposome Research. 2011;**21**(3):213-20

[19] Sekhon BS. Supercritical fluid technology: an overview of pharmaceutical applications. International Journal of PharmTech Research, 2010;**2**(1):810-26

[20] Jahn A, Vreeland WN, DeVoe DL, Locascio LE, Gaitan M. Microfluidic directed formation of liposomes of controlled size. Langmuir. 2007;**23**(11):6289-93

[21] Pons M, Foradada M, Estelrich J. Liposomes obtained by the ethanol injection method. International Journal of Pharmaceutics. 1993;**95**(1-3):51-6

[22] Yu B, Lee RJ, Lee LJ. Microfluidic methods for production of liposomes. Methods in Enzymology. 2009;**465**:129-41

[23] Avvaru B, Patil MN, Gogate PR, Pandit AB. Ultrasonic atomization: effect of liquid phase properties. Ultrasonics. 2006;**44**(2):146-58

[24] Kull H-J. Theory of the Rayleigh-Taylor instability. Physics Reports. 1991;**206**(5):197-325

[25] Abramov OV. High-intensity Ultrasonics: Theory and Industrial Applications: CRCPress; 1999, Amsterdam, The Netherlands

[26] Huang X, Caddell R, Yu B, Xu S, Theobald B, Lee LJ, et al. Ultrasound-enhanced microfluidic synthesis of liposomes. Anticancer Research. 2010;**30**(2):463-6

[27] Woodbury DJ, Richardson ES, Grigg AW, Welling RD, Knudson BH. Reducing liposome size with ultrasound: bimodal size distributions. Journal of Liposome Research. 2006;**16**(1):57-80

[28] Barba A, Bochicchio S, Lamberti G, Dalmoro A. Ultrasonic energy in liposome production: process modelling and size calculation. Soft Matter. 2014;**10**(15):2574-81

[29] Helfrich W. The size of bilayer vesicles generated by sonication. Physics Letters A. 1974;**50**(2):115-6

[30] Helfrich W. Elastic properties of lipid bilayers: theory and possible experiments. Zeitschrift für Naturforschung Teil C: Biochemie, Biophysik, Biologie, Virologie. 1973;**28**(11):693

[31] Fromherz P. Lipid-vesicle structure: size control by edge-active agents. Chemical Physics Letters. 1983;**94**(3):259-66

[32] Lasic DD, Papahadjopoulos D. Medical Applications of Liposomes: Elsevier; 1998, Amsterdam, The Netherlands

[33] Phenomena T. by RB Bird, WE Stewart, and EN Lightfoot. John Wiley, New York; 1960

[34] Bochicchio S, Dalmoro A, Recupido F, Lamberti G, Barba AA, "Nanoliposomes production by a protocol based on a simil-microfluidic approach", in "Lecture Notes in Bioengineering (LNBE)", Springer Ed.; 2017, Berlin, Germany

[35] Jahn A, Vreeland WN, Gaitan M, Locascio LE. Controlled vesicle self-assembly in microfluidic channels with hydrodynamic focusing. Journal of the American Chemical Society. 2004;**126**(9):2674-5

[36] Bochicchio S, Dapas B, Russo I, Ciacci C, Piazza O, De Smedt S, Pottie E, Barba AA, Grassi G, "In vitro and ex vivo delivery of new designed siRNA-nanoliposomes for E2F1 silencing as a potential therapy for Inflammatory Bowel Diseases-associated colorectal cancer". IJP—International Journal of Pharmaceutics, 2017 in press, doi.org/10.1016/j.ijpharm.2017.02.020

[37] Ibraheem D, Elaissari A, Fessi H. Gene therapy and DNA delivery systems. International Journal of Pharmaceutics. 2014;**459**(1):70-83

[38] Kim H-K, Davaa E, Myung C-S, Park J-S. Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid. International Journal of Pharmaceutics. 2010;**392**(1):141-7

[39] Fang Z, Gong C, Liu H, Zhang X, Mei L, Song M, et al. E2F1 promote the aggressiveness of human colorectal cancer by activating the ribonucleotide reductase small subunit M2. Biochemical and Biophysical Research Communications. 2015;**464**(2):407-15

[40] Bochicchio S, Dalmoro A, Barba A, d'Amore M, Lamberti G. New preparative approaches for micro and nano drug delivery carriers. Current Drug Delivery. 2017;**14**(2):203

[41] Dapas B, Farra R, Grassi M, Giansante C, Fiotti N, Uxa L, et al. Role of E2F1–cyclin E1-cyclin E2 circuit in human coronary smooth muscle cell proliferation and therapeutic potential of its downregulation by siRNAs. Molecular Medicine. 2009;**15**(9-10):297

[42] Poliseno L, Evangelista M, Mercatanti A, Mariani L, Citti L, Rainaldi G. The energy profiling of short interfering RNAs is highly predictive of their activity. Oligonucleotides. 2004;**14**(3):227-32

[43] Chithrani BD, Chan WC. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Letters. 2007;**7**(6):1542-50

[44] Mellican RI, Li J, Mehansho H, Nielsen SS. The role of iron and the factors affecting off-color development of polyphenols. Journal of Agricultural and Food Chemistry. 2003;**51**(8):2304-16

[45] Mehansho H. Iron fortification technology development: new approaches. The Journal of Nutrition. 2006;**136**(4):1059-63

[46] Mora JO. Iron supplementation: overcoming technical and practical barriers. The Journal of Nutrition. 2002;**132**(4):853S-5S

[47] Abbasi S, Azari S. Efficiency of novel iron microencapsulation techniques: fortification of milk. International Journal of Food Science & Technology. 2011;**46**(9):1927-33

[48] Stekel A, Monckeberg F, Beyda V. Combating iron deficiency in Chile: a case study: International Life Sciences Institute-Nutrition Foundation, 1986, Washington, D.C., USA

[49] Stekel A, Olivares M, Pizarro F, Chadud P, Lopez I, Amar M. Absorption of fortification iron from milk formulas in infants. The American Journal of Clinical Nutrition. 1986;**43**(6):917-22

[50] Lynch SR, Stoltzfus RJ. Iron and ascorbic acid: proposed fortification levels and recommended iron compounds. The Journal of Nutrition. 2003;**133**(9):2978S-84S

[51] Xia S, Xu S. Ferrous sulphate liposomes: preparation, stability and application in fluid milk. Food Research International. 2005;**38**(3):289-96

[52] Kosaraju SL, Tran C, Lawrence A. Liposomal delivery systems for encapsulation of ferrous sulphate: preparation and characterization. Journal of Liposome Research. 2006;**16**(4):347-58

[53] De Paoli T, Hager AA. Liposomes containing bioavailable iron [II] and process for obtaining them. Google Patents; 1996

[54] Danino D, Livney YD, Ramon O, Portnoy I, Cogan U. Beta-casein assemblies for enrichment of food and beverages and methods of preparation thereof. Google Patents; 2014

[55] Desai MP, Labhasetwar V, Amidon GL, Levy RJ. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharmaceutical Research. 1996;**13**(12):1838-45

[56] Hussain N, Jaitley V, Florence AT. Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. Advanced Drug Delivery Reviews. 2001;**50**(1):107-42