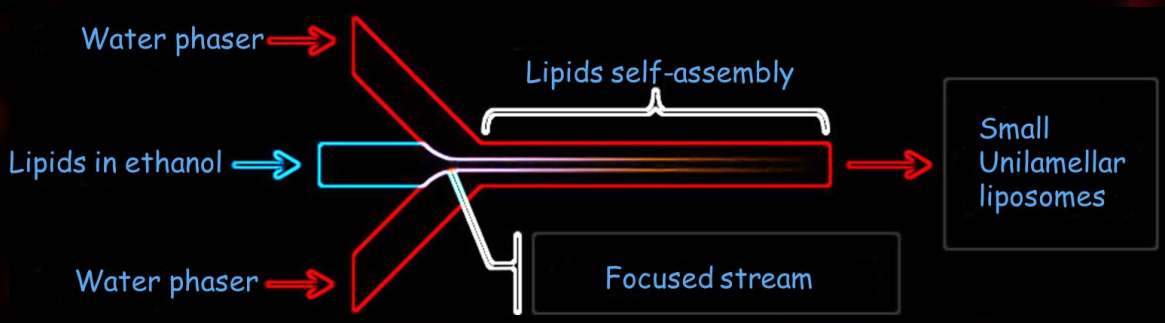
Produzioni di nanoliposomi per applicazioni nutraceutiche mediante un approccio simil-microfluidico



Federica Recupido

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Produzioni di nanoliposomi per applicazioni nutraceutiche mediante un approccio simil-microfluidico

Tesi in

Transport Phenomena in Food Processes

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Alla mia famiglia

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Sommario

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Abstract

Lipid-based delivery systems are biocompatible, safe and efficacious carriers, widely used for their capability in encapsulating and releasing, in a controlled manner, of sensible bioactive compounds for both pharmaceutical and nutraceutical applications. In particular, liposomes have attracted a lot of attention for their biodegradability, elevated drug loading, low intrinsic toxicity and easiness of preparation. In nutraceutical field, liposomes are used for the delivery of nutritional compounds with relevant healthy properties, such as vitamins, mineral salts, antioxidants, probiotics, enzymes and polyunsaturated fatty acids. Liposomes size and size distribution are key parameters for the improvement of nutraceuticals solubility, bioavailability and uptake and to preserve food product sensorial features (such as taste, aroma, flavor, appearance and consistence). Nowadays there is a wide set of possibilities to produce lipid-based drug delivery systems through the use of conventional or more recently discovered techniques. However, the majority of these methods are characterized by high energy request, long times of process together with a low productivity. In particular, the most used techniques, such as *ethanol injection*, are bench-scale methods characterized by bulk and discontinuous processes. Microfluidics is a relatively new technology used for the production of liposomes. This technique gives the possibility to produce, in a continuous manner, unilamellar nanometric liposomes by monitoring the micrometric fluid fluxes. Tuning the flow rates, thus controlling the lipids solution/buffer dilution process, liposomes dimension can be

controlled. Anyway, these methods are characterized by elevated costs of microfabrication and the low product volumes in output.

The aim of the thesis is to produce iron encapsulating nanoliposomes for nutraceutical applications. For this purpose, a bench-scale simil-microfluidic set up has been designed and developed. The apparatus basically consists in the realization of a contact between two flows, lipids/ethanol and water solutions, inside a millimetric tubular devices where interdiffusion phenomena allow the formation of lipid vesicles. Effects of solutions flow rates and lipids concentrations on size and size distribution have been investigated. Moreover, ultrasonic energy was used to enhance the homogenization of the hydroalcoholic final solutions and to promote the vesicles size reduction. By using the simil-microfluidic set up, liposomes encapsulating iron were produced by considering two formulation protocols in order to optimize the encapsulation efficiency. Then, a comparison in terms of vesicles final properties (mean diameter size, polidispersity index, zeta potential, charge and encapsulation efficiency) was realized between liposomes obtained by using the simil-microfluidic set up and the ones obtained using the conventional techniques (*ethanol injection* and *thin film hydration*) at the same conditions. Moreover, in order to perform propaedeutic studies for scaling up the plant, the possibility to produce liposomal structures using sonication batch with higher volumes (from few to hundreds of milliliters) was verified.

The most important results obtained from the research activities are the following. It was observed that by increasing the ratio between water solution volumetric flow rate and ethanol/lipids solution flow rate, liposomes size decreased; while, at fixed volumetric flow rates ratio, by increasing lipid concentration, vesicles size increased. Iron encapsulating liposomes are stable with mean diameter slightly higher than 100 nm; moreover, through the optimized formulation, an encapsulation efficiency of 97 % was achieved. Moreover, liposomes obtained by using the simil-microfluidic apparatus are comparable with the ones obtained with the conventional bench-scale techniques in terms of final characteristics, but, using the simil-microfluidic set up, process time reduces and

process yield increases thus achieving a massive nanoliposomes production. Then, sonication in duty-cycle, used for enhancing the homogenization of the hydroalcoholic bulk, was detected to be an efficacious and scalable technique in order to reduce vesicles size.

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Appendice

Parte dei risultati conseguiti con le attività condotte in questo lavoro di tesi sarà presentata al workshop internazionale BIONAM 2016 che si terrà in Salerno dal 6 al 7 ottobre 2016:

Sabrina Bochicchio, Annalisa Dalmoro, **Federica Recupido**, Gaetano Lamberti, Anna Angela Barba: "*Nanoliposomes production by a protocol based on a simil-microfluidic approach*", to be presented to the International Workshop BIONAM 2016 Salerno Italy 6^{th}-7^{th} October 2016- and in proceedings on "Lecture Notes in Bioengineering (LNBE)", Springer Ed.

Nanoliposomes production by a protocol based on a simil-microfluidic approach

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Abstract. In this work a protocol based on the microfluidic principles has been developed and applied to produce nanoliposomes. The protocol basically consists in the realization of a contact between two flows, lipids/ethanol and water solutions, inside a tubular device where interdiffusion phenomena allow the formation of lipid vesicles. Effects of solutions flow rates and lipids concentrations on size and size distribution have been investigated. Moreover ultrasonic energy was used to enhance homogenization of the hydroalcoholic final solutions and to promote the vesicles size reduction. By this protocol a massive output has been achieved; increasing the ratio between the water volumetric flow rate to the lipids-ethanol volumetric flow rate the liposomes dimension decreases; at equal flow rates, when the lipids concentration increases also the liposomes size has been observed increasing.

1 Introduction

Lipid-based drug delivery systems are biocompatible, safe and efficacious carriers even more investigated by the scientific world for their ability in encapsulating and releasing, in a controlled manner, degradable active ingredients to be used for pharmaceutical and nutraceutical purposes. In particular liposomes have attracted a lot of attention for their biodegradability, high drug loading, low intrinsic toxicity, accumulation in pathological areas, reduced size, membrane mimetic behavior, prolonged half-life in the bloodstream, low cost and easiness of preparation [1]. In particular, size and size distribution are key parameters determining liposomes performance as carrier systems in both biomedical applications (i.e. influencing liposomes time of circulation in the blood stream and/or their permeability through membrane fenestration in tumour blood vessels [2]) and nutraceutical applications (i.e. improving taste, flavor, stability, absorption and bioavailability of nutraceuticals [3-4]). Nowadays there is a wide set of possibilities to produce lipid-based drug delivery systems through the use of conventional or more recently discovered techniques [5-7]. 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 together with a low productivity.
To overcome these limitations, in this study microfluidics based methods, which are expensive for special devices needed and microfabrication costs, have been transposed to a millimeter scale, drastically reducing the production costs and increasing the yields. With the aim to have a control on flow, typically chaotic in a bulk phase which is instead laminar, and thus controllable, in a microfluidic system, starting from a work of Pradhan and collaborators [8], in which a syringe pump driven microfluidic device was used to produce liposomes, the design and the developed of a new semi-continuous bench scale apparatus for a massive nanoliposomes production, overcoming the limits imposed by the syringe volumes, has been done. The preparative protocol pointed out basically consists in the realization of a contact between two flows, lipids/ethanol and water solutions, inside a tubular device where interdiffusion phenomena allow the formation of lipid vesicles. Ultrasonic energy was also used as intensification tool for liposomes production, size reduction and homogenization [9]. Furthermore in this work, similarly as done by Jahn and collaborators for a microfluidic hydrodynamic focusing (MHF) platform [10], a size and size distribution control of the produced nanoliposomes was demonstrated by tuning not only the flow rates, as done by Jahn research group, but also the lipids concentration.

2 Experimental

2.1 Materials and Methods

Liposomes were formulated with L-α-Phosphatidylcholine (PC) from soybean, Type II-S, 14-23% choline basis (CAS n. 8002-43-5), purchased from Sigma Aldrich (Milan, Italy) and ethanol of analytical grade (CAS n. 64-17-5, Sigma Aldrich) was used to solubilize the PC. Rhodamine B was used for microscope fluorescence observations of the produced liposomes.
In Figure 1 a schematic representation of the used experimental apparatus is presented. The first part of the bench-scale apparatus consists in two containers, one filled with a lipids/ethanol solution (prepared by dissolving 16.5 mg of PC in 10 ml of ethanol), which is conveyed in a 1.6 mm silicon tube, and one filled with the hydration solution (distilled water) conveyed in a 5 mm silicon tube through the use of two peristaltic pumps (Verderflex OEM mod. Au EZ). The lipids/ethanol solution tube ends with a needle (0.6 mm internal diameter) inserted into the production section tube, a 3 mm internal diameter silicon tube which is an extension of the water tube. This is the production section, where a diffusive mechanism of the two pushed liquids takes part leading to the formation of liposomes during the two phases (water and alcohol) interdiffusion. The hydroalcoholic solution containing liposomes on nanometric scale is recovered in a container and then subjected to a duty cycle sonication process in order to homogenize and to further reduce vesicles size.

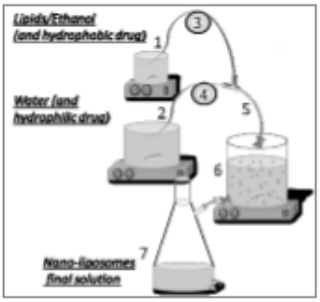

Fig. 1. Schematization of the simil-microfluidic apparatus. From the containers (1-2) lipids/ethanol and water solutions are pushed through peristaltic pumps (3-4) to the production section (5) were vesicles are formed. The hydroalcoholic solution is recovered in a container (6) where it is stirred. The nanoliposomes solution is finally subjected to duty cycle sonication and recovered in a receiving flask (7).

At first, liposomes were prepared by maintaining constant the PC concentration in the hydroalcoholic solution at 0.15 mg/ml and varying the volumetric flow rates in order to study their influence on liposomes size and size distribution. The volumetric flow rates ratio, defined as hydration solution volumetric flow rate (Vhs) to lipid solution volumetric flow rate (Vls), was varied (10:1, 15:1, 20:1 and 40:1 Vhs/Vls) maintaining constant the Vls at 4 ml/min and changing the Vhs (40 ml/min, 60 ml/min; 80 ml/min and 160 ml/min). Subsequently, the PC concentration in the final hydroalcoholic solution was changed (0.15, 1, 4 and 5 mg/ml) by maintaining constant at 10:1 (Vhs/Vls) the volumetric flow rate ratio, and the liposomes size and size distribution were analyzed again. Part of all the produced samples was then subjected to a sonication process previously developed and modelled by Barba and collaborators [9] by applying a duty cycle consisting of 5 ten-second irradiation rounds each followed by a 20 second pause in order to prevent thermal vesicle disruption (VCX 130 PB Ultrasonic Processors, Sonics & Materials Inc., Newtown, CT, USA). For both the experimental campaigns the volumetric flow rate ratio and PC concentration effects on liposomes size and size distribution were studied with and without the ultrasound contribution.

Morphological characterizations of liposomes were performed using optical microscopy (Axioplan 2- Image Zeiss) for fluorescence imaging, a 100 X oil immersion objective was used to visualize the vesicles. Dimensional characterization of vesicles was performed by Dynamic Light Scattering analysis (Zetasizer Nano ZS, Malvern, UK). The resulting particle size distribution was plotted as number of liposomes versus size. The Polydispersity Index (PDI) was calculated for all the preparations.

3 Results and Discussion

3.1 Nanoliposomes production

The developed set up has proven to be an easy and fast method to produce Small Unilamellar Vesicles (SUVs) directly on a nanometric scale, without the use of any toxic solvents and drastic operative conditions (i.e. high temperature and/or pressure) achieving a massive output with the minimum of energy and costs. In Figure 2 fluorescence microscopy images of obtained SUVs are shown.

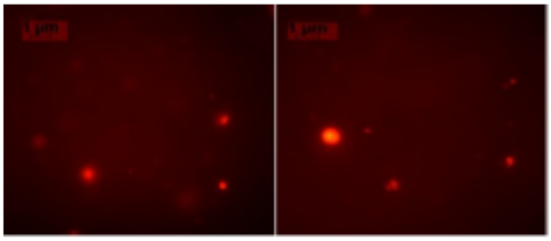

Fig. 2. Fluorescence microscopy images of lipid vesicles labelled with Rhodamine B dye and visualized with a 100 X objective.

From a phenomenological point of view, liposomes formation happens at the interfaces between the alcoholic and water phases, when they start to interdiffuse in a direction normal to the liquid flow stream. When lipids, dissolved in the alcoholic phase, meet with water, due to their insolubility, they start to assemble together generating pieces of bilayer which close themselves engulfing water and forming spherical vesicles. Even if the liposomes formation is a spontaneous process, their dimension and size distribution as well as the productivity of the process (the number of liposomes for unit volume obtainable) can be controlled operating on the volumetric flow rates ratio of the two liquids, on lipids concentration and using ultrasonic energy as tool for the process intensification.

3.2 Influence of volumetric flow rate ratio on liposomes formation

As reported in Figure 3, increasing the ratio between the water volumetric flow rate to the lipids-ethanol volumetric flow rate the liposomes dimension decreases (Figure 3-A) while the PDI value increases (Figure 3-B). In particular, starting with a 10:1 Vhs/Vls ratio, liposomes of 49 nm in size were obtained up to a diameter of 33 nm for liposomes achieved with 40:1 Vhs/Vls, the higher volumetric flow rate ratio tested. Viceversa opposite trend is visible for the PDI whose 0.36 value obtained from the 10:1 Vhs/Vls becomes 0.73 when a 40:1 Vhs/Vls ratio was used, index, the latter, of an highly polydispersed sample. Taking into account that the Vhs/Vls ratio was increased by enhancing the water flow rate and maintaining constant that of the lipids-ethanol solution, what plays a key role in the liposomes diameter size reduction is the diffusion rate of the lipids into the hydration solution. Considering constant and uniform the lipids concentration for unit of volume in the alcoholic solution, when the

water volumetric flow rate increases the impact between the two liquids increases too and lipids spread faster in a greater water volume. Lipids so scattered join together to form vesicles even more smaller with the increase of water flow rate. For both size and size distribution it can be observed that the ultrasound assisted process applied not only reduce the liposomes size for all the volumetric flow rates explored but it does so by maintaining about the same decreasing trend found for the not-sonicated sample (Figure 3-A). It was demonstrated that sonication can reduce liposomes size up to a maximum of 31% (at 40:1 Vhs/Vls ratio) respect to the not-sonicated liposomes diameter size. Moreover, the duty cycle sonication has a key role in the liposomes homogenization by reducing the polydispersity of the sample, optimizing the size distribution in all the condition tested; for the 40:1 Vhs/Vls the PDI is nearly halved (Figure 3-B).

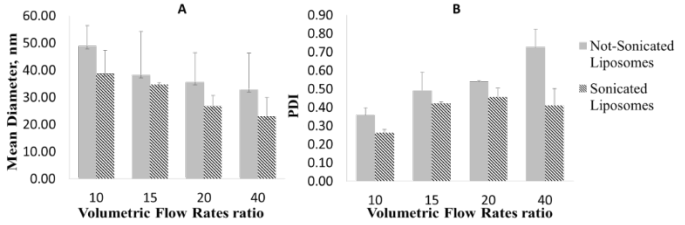

Fig. 3. A) Sonicated and not sonicated liposomes mean diameter size at different volumetric flow rates ratio. B) Polydispersity Index (PDI) of sonicated and not sonicated liposomes at different volumetric flow rates ratio. Results are expressed as average of three determinations and reported along with the standard deviation.

3.3 Influence of lipid concentration on liposomes formation

It was observed that at equal flow rates, when the lipids concentration increases also the liposomes size has risen. In Figure 4-A data shown illustrate liposomes diameter average size obtained at different PC concentrations in the hydroalcoholic solution. As the PC concentration increases from 0.15 mg/ml to 5 mg/ml the liposomes size also increases from approximately 49 nm to 81 nm. It can be explained by the fact that at higher PC concentrations more phospholipids will impact at the same alcohol/water interface area and will dissolved in the same water volume making them physically closer to each other, thus forming larger vesicles. For PDI values it seems that increasing the PC concentration the sample size distribution improves except for the 0.15 mg/ml concentration (Figure 4-B). The sonication process has confirmed to be absolutely efficacy for both liposomes size reduction and homogenization as already seen for liposomes production at different volumetric flow rate ratio.

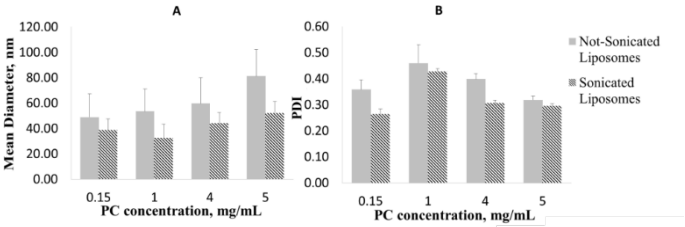

Fig. 4. A) Sonicated and not sonicated liposomes diameter size at different PC concentrations in the hydroalcoholic solution. B) Polydispersity Index (PDI) of sonicated and not sonicated liposomes at different PC concentrations in the hydroalcoholic solution. Results are expressed as average of three determinations and reported along with the standard deviation.

4 Conclusions

Nanoliposomes have been achieved by using a protocol based on the microfluidic principles. Effects of solutions flow rates and lipids concentrations on size and size distribution have been investigated. Increasing the ratio between the water volumetric flow rate to the lipids-ethanol volumetric flow rate the liposomes dimension decreases; at equal flow rates, when the lipids concentration increases also the liposomes size has been observed increasing. Ultrasonic energy was used to enhance homogenization of the hydroalcoholic bulk and, as expected on the bases of previous studies, its duty cycle application efficaciously promoted vesicles size reduction.

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