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Nanostructured vectors for the transport of active molecules through biological membranes for pharmaceutical and nutraceutical applications

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Abstract PhD

Purpose of the PhD thesis was to develop dedicated lipid nanostructured vectors with tailored features (in terms of size, surface charge, load capability, stimuli responsive ability and stability) through the design of novel production processes expressly developed for nutraceutical and therapeutic agents encapsulation.
The preliminary performed review of the main processes used for liposomes production have underlined that the majority of the conventional and more innovative methods adopted show a number of drawbacks such as few product volumes in output (directly linked to the impossibility in scaling up the process), high energy consumption, long times of production together with the use of toxic solvents and other process drastic conditions. To the light of these literature findings and with the aim to produce nanostructured vectors through more sustainable processes, two novel techniques, sharing the ultrasound technology as process intensification tool used in particles size reduction and homogenization operations, were designed and developed to respond to the needs of a better process performance, improving its efficiency and cutting down energy consumption.
At first, based on the use of ultrasound as alternative energy resource, a solid particles size reduction process was developed and coupled with the bench scale conventional Thin Film Hydration (TFH) method. This technique provides the generation of a lipid film which is formed after solvents evaporation through the use of a rotary evaporator. The dried film is then hydrated, spontaneously producing micrometric vesicles characterized by the presence of several bilayers. Then the method was revisited by adding the ultrasound assisted step developed in order to produce, in a versatile manner, structures with the desired dimension (on micro/nano scale), starting from the micrometric ones.
Four are the main sections composing the set-up to apply this innovative protocol: a feeding section, a solvent evaporation section, a liposomes production/homogenization section and a recovery section. In particular, the homogenization section is composed of a 3 mm sonication tip (operative frequency 20 kHz) which acts on micrometric vesicles sample aliquots.
Subsequently to the realization of the production bench scale apparatus, the phenomenology connected to the vectors constitution was investigated and a dynamic model able to describe the curvature of a lipid bilayer under the effect of ultrasonic energy was then proposed and tested.
In that regard, starting from micrometric vesicles, the ultrasound energy is used to break the lipid bilayer into smaller pieces, then these pieces close themselves in spherical structures producing small vesicles. Moreover the role of several process parameters were also elucidated.
Once established its reliability and due its great potential in reducing time spent, without compromising the integrity of the liposomal systems produced (in terms of structure and load), the ultrasound intensification tool was also used for liposomes homogenization operation during vesicles production through a simil-microfluidic approach.
As a matter of fact, in order to produce higher volumes of lipid vectors, potentially on production scale, directly with nanometric size, a simil-microfluidic apparatus was expressly designed and fabricated, overcoming the limitations of the small output volumes typical of the conventional bench scale techniques.
There are five main sections composing the realized apparatus: a feeding section, a pumping section, a production section, an homogenization section and a recovery section. In particular the homogenization section is composed of a 6 mm sonication tip (operative frequency 20 kHz) directly immersed in the entire hydroalcholic solution containing nanoliposomes.
As previously done, the phenomenological aspects involved in vectors constitution were investigated for this new adopted set-up. In particular, the reproduction of the phenomenology connected to the vesicles formation through a microfluidic approach was achieved by the use of constructive expedients (millimetric diameter of tubes, peristaltic pumps, injection needle). Particularly, nanostructured vectors 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; changes in flow conditions result in size variations of the insertion section of the organic phase reflecting on the vesicles dimensional features.

In that regards, taking into account that size and size distribution are key parameters determining liposomes performance as carrier systems in both pharmaceutical and nutraceutical applications, a control on the produced nanoliposomes dimensional features was demonstrated by tuning the volumetric flow rates and the lipids concentration process parameters. In particular, it was understood that increasing the ratio between the water volumetric flow rate to the lipids-ethanol volumetric flow rate the liposomes dimensional distibution increases; on contrary, ultrasonic energy enhances the homogenization of the hydroalcoholic bulk and, as expected on the bases of previous studies conducted on smaller volumes, its duty cycle application efficaciously promoted a better vesicles dimensional distribution. This result was also confirmed by working at equal flow rates but at different lipid concentrations. Finally, the developed simil-microfluidic apparatus, working at room conditions and in absence of toxic solvents, makes nanoliposomes production a safe and low cost process, highly productive due to the use of ultrasound which was demonstrated to be a scalable means for process intensification. By using the two developed experimental set-up, several classes of liposomal structures were formulated and produced to respond to specific requests of nutraceutical and pharmaceutical applications. Through the ultrasound assisted tool at first coupled with the conventional THF method and subsequently used as integrant part of the homogenization section of the simil-microfluidic apparatus, different active molecules were successfully encapsulated in lipid nanostructured vectors solving the critical issues linked to their naked administration and transport through biological membranes. In particular, nanoliposomes containing vitamins with different hydrophobicity (α-tocopherol, ergocalciferol, vitamin B12) and ferrous sulfate, with highly interesting features for nutraceutical market, were produced achieving stable loaded nanoliposomes with high encapsulation efficiencies and good dimensional features.

In details, for vitamins-nanoliposomes productions, neuter vesicles with micrometric size, ranging from 2.9 μm to 5.7 μm, were produced, obtaining, after sonication in duty cycle, small vesicles in the average range of 40 nm to 51 nm in size. High encapsulation efficiency (e.e.) was obtained in both micrometric vesicles, with a e.e. % of 72.0 ± 00 % for vitamin B12, 95.0 ± 7.07 % for α-tocopherol and 81.5 ± 2.12 % for ergocalciferol, and small vesicles, with an e.e. % of 56.2 ± 8.51 % for vitamin B12, 76.3 ± 14.02 % for α-tocopherol and 57.5 ± 13.9 % for ergocalciferol (the higher the vitamin hydrophobicity, the higher the encapsulation efficiency). Finally, a comparison between vitamin B12 load achievable with the developed technique and the vitamin load achievable by breaking unloaded preformed liposomes (conventional approach) showed an increase of encapsulation efficiency in small vesicles from 40% to 56.2 %, confirming the effectiveness of the pointed out technique.

Regarding the ferrous sulfate-nanoliposomes, their massive production was possible due to the simil-microfluidic approach with a precise control on particles size and size distribution. In particular, the effect of different weight ratios of iron to the total formulation components (0.06, 0.035, 0.02 and 0.01 iron/total components weight ratio) on the final vesicles encapsulation efficiency was investigated obtaining with the last formulation an high encapsulation efficiency (up to 97%).

In general, ferrous sulfate loaded nanoliposomes, negative charged, with good dimensional features (127-135 nm for not sonicated and 48-76 nm for sonicated liposomes) were successfully produced through the use of the simil-microfluidic method developed, obtaining an elevated process yield if compared to the classical bench scale techniques (THF and Ethanol Injection).

For pharmaceutical purposes, anionic nanoliposomes containing a new synthetized peptide (KRX29) for a not conventional heart failures therapy and new, cutting edge, nucleic acids based therapeutics agents (NABDs), used in gene therapy, were successfully produced.

Regarding KRX29-nanoliposomes production, micrometric particles of 7.2-11.7 µm were obtained and sized with the use of the developed ultrasound assisted process thus achieving 22 – 35 nm vesicles. The effect of liposomes charge on both peptide encapsulation and recovery efficiencies was at first studied, showing an higher encapsulation efficiency (about 100%) achieved (both in small and large vesicles) by using the higher charge ratio formulation (13:1 (-/+)). Viceversa, the ability to recover the entrapped peptide was obtained for loaded systems (both in small and large vesicles) at the lower charge ratio formulation (1:1 (-/+)). As the charge ratio, also the peptide concentration showed influence on the liposomes encapsulation efficiency.
For NABDs complexes production, at first preliminary experiments in which dsDNA was used to simulate the structure of siRNA molecule were done by testing different dsDNA/DOTAP lipid charge ratio (3:1, 5:1 and 7:1 (+/-)) in order to achieve the higher dsDNA encapsulation efficiency in the smaller carrier possible. DOTAP phospholipid was used due to its positive charge. The performed activities have confirmed the versatility of the ultrasound assisted technique for producing micro (2.2 – 2.9 µm) and nano lipid vectors (28 - 56 nm) encapsulating NABDs. In particular, the charge ratio (+/-) variation from 3:1 to 7:1 (+/-) by changing the amount of positive lipid (DOTAP) used in liposome preparation have allowed to an improved e.e. wich was 64 % and 100 % respectively for small and large vesicles by using the 7:1 (+/-) charge ratio.
Starting from these preliminary tests, siRNAs-nanoliposomes complexes were produced for the inhibition of E2F1 protein expression, studied as a potential way to treat colorectal cancer associated to Inflammatory Bowel Diseases. By the TFH/sonication technique nanoliposomes with 33-38 nm range size and 100% siRNA encapsulation efficiency were obtained. The produced loaded nanoliposomes 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 anti-E2F1 expression effect after siE2F1-1324-nanoliposome samples transfection has been demonstrated also in a dynamic human model such the colon tissue microenvironment (i.e. an 80.5% reduction of E2F1 expression respect to the basal tissue was achieved in patient 4), a clear tendency to respond in a patient-dependent way was observed.

All the achieved results highlight the potentiality of the purposely designed nanoliposomes in deliver, in a controlled manner, different active molecules for both pharmaceutical and nutraceutical purposes. The formulative and the chemical engineering approaches adopted in this thesis for nanostructured vectors production respectively enhance the product quality (nanoparticles with tailored features) and make the process more attractive in terms of improved safety and reduced costs.