MICROFLUIDIC ASSEMBLY OF 'TURTLE-LIKE' SOLID LIPID NANOPARTICLES FOR LYSOZYME DELIVERY

F. Sommonte,[1,#] I. Arduino,[1,#] R.M. Iacobazzi,[1] M. Tiboni,[2] F. Catalano,[3] R. Marotta,[3] M. Di Francesco,[4] L. Casettari [2], P. Decuzzi,[4] A. Lopedota[1] and N. Denora[1,*]

[1] Department of Pharmacy - Pharmaceutical Sciences, University of Bari "Aldo Moro", Orabona St. 4, 70125, Bari, Italy.

[2] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Piazza del Rinascimento 6, 61029, Urbino, Italy.

[3] Electron Microscopy Facility, Fondazione Istituto Italiano di Tecnologia, Morego St. 30, 16163, Genoa, Italy.

[4] Laboratory of Nanotechnology for Precision Medicine, Fondazione Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genoa, Italy.

[#] F.S. and I.A. have contributed equally and share the first authorship.

* Corresponding author: Nunzio Denora, PhD, nunzio.denora@uniba.it

ABSTRACT

After two decades of research in the field of nanomedicine, nanoscale delivery systems for biologicals are becoming clinically relevant tools. Microfluidic-based fabrication processes are replacing conventional techniques based on precipitation, emulsion, and homogenization. Here, the focus is on solid lipid nanoparticles (SLNs) for the encapsulation and delivery of enzymes. A thorough analysis was conducted to compare conventional versus microfluidic-based production techniques, using a 3D printed device. The efficiency of the microfluidic-based technique in producing Lysozyme-loaded SLNs (LZ SLNs) was demonstrated: LZ SLNs were found to have a lower size (158 ± 4.86 nm vs 180 ± 7.46 nm) and higher encapsulation efficiency (70 ± 1.65 % vs 53.58 ± 1.13 %) as compared to particles obtained with conventional methods. Cryo-TEM studies highlighted a peculiar turtle-like structure on the surface of the LZ SLNs. In vitro studies demonstrated that LZ SLNs were suitable for achieving a sustained release over time (7 days) at the site of interest. The enzymatic activity of LZ entrapped into SLNs was challenged on *Micrococcus Lysodeikticus* cultures, confirming the stability and potency of the biologicals. This systematic analysis demonstrates that microfluidic production of SLNs can be efficiently and effectively used to encapsulation and delivery complex biological molecules.

Keywords: Microfluidics; Additive manufacturing; Drug delivery systems; Biologicals delivery; Lipid-based nanocarriers

Abbreviations: SLNs, Solid Lipid Nanoparticles; LZ, Lysozyme; LZ SLNs, Lysozyme-loaded Solid Lipid Nanoparticles; FDM, Fused Deposition Modeling; CAD, Computer Aided Design; TFR, Total Flow Rate; FRR, Fluid Rate Ratio; TEM, Transmission Electron Microscope; Cryo-EM, Cryogenic Electron Imaging; Encapsulation Efficiency, EE.

1. Introduction

In the last decade, nanosized drug delivery systems have attracted the focus of the scientific community for a variety of pharmaceutical applications (Anselmo et al., 2018). The open literature has extensively documented that colloidal nano-systems offer specific advantages in drug delivery and imaging over the native free molecules. Indeed, nanoparticulate systems enable the controlled and extended release of a variety of hydrophilic and hydrophobic agents, allow for the specific deposition of the payloads within the diseased tissue, reduce systemic toxicity associated with off-target drug accumulation, support the co-delivery of multiple agents realizing complex therapeutic regimens (combination therapies) and fusing therapeutic intervention with monitoring (theragnostic) (Arduino et al., 2021b; Svenson, 2012). Lately, several therapeutic protocols involve the use of biological actives also referred to as biologicals, defined by World Health Organization as an emerging class of medicine produced and purified from large-scale cell cultures of bacteria or yeast, or plant or animal cells, and which includes vaccines, growth factors, immune modulators, monoclonal antibodies, as well as products derived from human blood and plasma (World Health Organization, 2022). However, upon systemic administration, biologicals may undergo enzymatic degradation and experience difficulties in crossing restrictive biological barriers due to their large size (Anselmo et al., 2018). Therefore, their use in therapy is still challenging. Furthermore, biologicals are more unstable than small molecules and their manipulation during formulation and storage could lead to chemical-physical degradation. Thus, there is a growing need to design adequate drug delivery systems that could enable the safe and secure transport of biologicals preventing instability phenomena (Chiesa et al., 2021).

A promising and attractive approach involves solid lipid nanoparticles (SLNs) (Arduino et al., 2020a, 2021a, 2021b; Iacobazzi et al., 2022). This innovative drug delivery system may combine the advantages of other innovative formulations while enabling to exceed some of their limitations (Sommonte et al., 2021). In more detail, some of the features that have contributed to identifying SLNs as potential strategy compared to other drug delivery systems include: the high stability over

time, the ability to provide sustained release from days to weeks, the biocompatibility of the starting materials employed, the possibility to encapsulate both hydrophilic and hydrophobic drugs, and the great stability provided to the encapsulated active molecules into their solid lipid core (Gastaldi et al., 2014; Mehnert and Mader, 2001). Moreover, in terms of their application, SLNs are more commonly explored as solubilizing agents for the delivery of poorly soluble drugs due to the hydrophobic nature of the particle-matrix. However, they have also been investigated for the delivery of nucleic acids, proteins, antigens, or in the food industry as carriers for bioactive compounds or to protect biomolecules against degradation (Cerqueira et al., 2014; Shegokar et al., 2011; Weiss et al., 2008). This property of SLNs seemed to render this type of formulation suitable for the loading of active compounds that suffer chemical-physical instability processes. Thus, the idea of using lipidic nanoparticles as carriers for the delivery of sensitive macromolecules to specific target tissues.

Although novel formulations propose several advantages, their clinical use is still very limited. This dilemma could be explained by the fact that there is no classical production method that ensures rapid production, high batch-to-batch reproducibility, and easy scale-up (Arduino et al., 2021b). Specifically, evidence in the literature has shown that nanosystems smaller than 200 nm in size are skilled at crossing several biological membranes including the blood-brain barrier, which limits and prevents the circulation of bioactive substances into the brain (Sommonte et al., 2021). Hence, the urgent need to obtain monodisperse, narrow-size nanoformulations suitable for their potential development in therapy. Due to the previously mentioned reasons, it was considered appropriate to identify an alternative way to conventional manufacturing replacing bench-top methods. In recent years, the microfluidic technique has acquired a leading role in this area, as *in-flow* production has allowed overcoming many of the issues mentioned above (Gastaldi et al., 2014; Weaver et al., 2021). In fact, the microfluidic method involves the formulation of nanoparticulate systems, in our case SLNs, by nanoprecipitation in fluidic microchannels. The *in-flow* process allows to increase in the repeatability of the achieved results, obtaining nanosystems with narrower size distribution and higher batch-to-batch reliability. These improvements regarding classical production are due to the use of a

microfluidic device that allows the fine control of the concentrations of the reagents used, the temperature of the system, the total flow rate (TFR), and the ratios between the external and internal fluid flow rate (Chiesa et al., 2021; Martins et al., 2018; van Ballegooie et al., 2019). A fine optimization of the microfluidic parameters and the geometry of the device has allowed to control the final characteristics of different formulations, highlighting the great versatility and the feasibility of this technique for industrial scale-up (Teixeira et al., 2020; Webb et al., 2020).

In this work, Lysozyme (LZ), as a model enzyme, was incorporated into SLNs, and the bench-top preparation method was compared to the microfluidic technique. In line with a previous study in which an *in-house* fabricated glass device was developed, Arduino et al. (2021b) the focus of this project was to optimize the manufacturing process of LZ SLNs using a 3D-printed polypropylene device (Tiboni et al., 2021) with a different geometry compared to the previous work. Additionally, it has deeply investigated the SLNs morphology using negative staining Transmission Electron Microscope (TEM) and Cryogenic Electron Imaging (Cryo-EM) analysis highlighting, for the first time, the unique turtle-like structure of SLNs. Finally, the short- and long-term stability of the nanosystem was assessed, while tests on *Micrococcus Lysodeikticus* cell cultures were conducted to demonstrate the actual activity of the released enzyme from SLNs on its biological substrate.

2. Materials and methods

2.1 Materials

All chemicals were purchased at the highest available purity and used as received without further purification or distillation. Cetyl palmitate was provided by Farmalabor. Lysozyme (from chicken egg white, lyophilized powder, protein ≥ 90%, ≥ 40,000 units/mg protein) and human serum were purchased from Sigma-Aldrich. *Micrococcus Lysodeikticus* was purchased from Sigma-Aldrich (ATCC No. 4698, lyophilized cells M3770). Polypropylene was kindly donated by BASF. All

solvents were of analytical grade and purchased from Aldrich. Lutrol F68 (Poloxamer 188) was purchased from Sigma-Aldrich. Human serum was purchased from Sigma-Aldrich.

2.2 Chip's fabrication by 3D printing

The microfluidic devices were produced by fused deposition modeling (FDM) 3D printing as previously reported (Tiboni et al., 2021). Briefly, a passive micromixer based on "zigzag" bas-relief was designed with a computer aided design (CAD) software and then exported as STL (Stereolithography interface format) to be then converted in machine language with a computer aided manufacturing (CAM) software. The microfluidic devices were printed using polypropylene in a FDM 3D printer (Ultimaker 3, Ultimaker, The Netherlands) at a print speed of 25 mm/s with a nozzle temperature of 205 °C (0.25 mm nozzle). The infill density was set at 100% and the build plate was preheated at 85 °C. Probe needles were used to connect the chip with the pump tubing. The channels of the chip had a 1 mm square section while the zigzag structure had a height of 500 μm. The total length of the main channel was 60 mm.

2.3 Preparation of solid lipid nanoparticles via a bench-top method

SLNs were prepared by oil-in-water hot homogenization technique, according to literature (Arduino et al., 2020a; Iacobazzi et al., 2022a) with some modifications. In brief, 50 mg of cetyl palmitate were dissolved into 1 mL of chloroform. The organic phase was added drop by drop into an aqueous solution (ultrapure water, 3 mL), containing Lutrol F68 1,67% (w/v) at 60 °C, and sonicated for 15 minutes by using an ultrasound probe-tip (0.25 W). The organic phase was rapidly evaporated at 60 °C via a rotary evaporator. The formulation was left at room temperature for 2 h to promote the complete evaporation of the chloroform and then, kept at 4 °C for 15 min to allow the SLNs' formation. In the case of LZ-SLNs, the enzyme was added to the aqueous phase. Several concentrations of LZ were tested with a LZ-to-lipid ratio of 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25. The resulting preparation was subsequently purified by centrifugation using centrifuge filters (Amicon®

Ultra- 15, Centrifugal Filters Ultracel® - 50K, Merck Millipore Ltd., CORK IRELAND), and four washes with ultrapure water were performed (3,500 rpm, 5 min, 4 °C).

2.4 Preparation of solid lipid nanoparticles via a microfluidic technique

SLNs were produced by a nanoprecipitation process using a 3D printed polypropylene device manufactured as reported above (Tiboni et al., 2021). The chip had two separate inlets that converge with a T-junction into the main channel. The device is structured so that the nanoprecipitation process occurs due to the whirling mixing between internal and external fluids, which flow in a single direction. The miscible fluids were pumped into the microfluidic device through polyethylene syringes mounted on syringe pumps allowing to keep a constant flow rate. The fluid carrying the lipid matrix was a 95% ethanol solution, while the other fluid was an aqueous solution containing a stabilizing surfactant (*i.e.*, Lutrol F68 2%). The synthesis of SLNs was optimized by adjusting the Total Flow Rate (TFR) (i.e., the sum of the flow rates for the aqueous and organic solutions) and the Flow Rate Ratio (FRR) (*i.e.*, the rate between the flow rate of the aqueous solution and the flow rate of organic solution). The aqueous solution of Lutrol F68 2% (w/v) was filtered using CA syringe filter 25 mm 0.45 μm (Scharlab S.L., Barcelona, Spain). The lipid matrix was composed of cetyl palmitate (10 mg/mL) in 95% ethanol. The optimization process has involved several experimental tests to achieve monodispersed SLNs. The two fluids were injected into the device from separate inlets at FFR ranging from 0.2:8 to 5:25. During the process, it was fundamental to keep the temperature over the melting point of the lipid. In this regard, an infrared lamp (Incandescent 230-250V BR125, 150W E27 IR RE, PHILIPS) was positioned 10 cm from the syringe containing the organic phase, while the microfluidic device was placed in a hot bath with a fixed temperature above 60 °C during the entire manufacturing process. After synthesis, the SLNs were left on a hot stirring plate to facilitate the elimination of the organic solvent, then equilibrated to room temperature, and finally cooled for the consolidation process (4°C for 15 minutes). In the case of LZ-SLNs, the enzyme was added to the surfactant-stabilized aqueous phase. A final purification step was performed to

remove residual traces of the organic solvent and unencapsulated enzyme using centrifuge filters (Amicon® Ultra- 15, Centrifugal Filters Ultracel® - 50K, Merck Millipore Ltd., CORK IRELAND) and four washes with ultrapure water (3500 rpm, 5 minutes, 4°C) were conducted.

2.5 Evaluation of the enzyme encapsulation efficiency

The encapsulation efficiency (EE%) was assessed by quantifying the LZ content in 200 μL of SLNs. More specifically, LZ SLNs were digested using a 500 μL mixture containing n-hexane and ultrapure water (1:1 (v/v)). The amount of enzyme extracted in the aqueous phase was quantified by the Bradford method (Khramtsov et al., 2021). All absorbance measurements were carried out in triplicate at room temperature. The encapsulation efficiency (EE%) for the enzyme was calculated using Eq. (1) below:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{mass of LZ into SLN}}{\text{mass of LZ added initially}} \times 100 \quad (1)$$

2.6 Particle size, size distribution, and surface electrostatic charge

The size distribution and ζ-potential of the nanoparticles were evaluated using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Approximately 1 mL of a 1:50 diluted solution in ultrapure water of each sample was analyzed using disposable polystyrene cuvettes (Sarstedt AG & Co., Germany) at 25 ± 0.1 °C. The surface ζ-potential of the SLNs was evaluated using a 750 μL of the 1:50 dilution in demineralized water of the nanoparticle suspension in a disposable folder capillary cell (DTS1070, Malvern, UK). The resulting data are shown as the numerical mean and standard deviation of the measurements of different samples, each sample measured three times.

2.7 Transmission Electron Microscopy and Cryogenic Electron Imaging analyses

For the negative-staining TEM analysis, samples were diluted approximatively to 0.1 mg/mL in milli-Q water. Then, they were dropcasted onto ultrathin C 150 mesh Cu grids and stained with uranyl acetate 1% in water for 60 sec before being analyzed with a JEOL (JEM-1011 TEM, Japan), equipped with a thermionic source (W filament) operating at 100 kV. The images were acquired using a DigitalMicrograph (TM), version 1.71.38 Gatan Inc, Pleasanton, CA (USA). For Cryo-EM, sample vitrification was performed in liquid ethane cooled at liquid nitrogen temperature using the FEI Vitrobot Mark IV semiautomatic autoplunger. Bright field cryo-EM was run at −176 °C in a FEI Tecnai G2 F20 transmission electron microscope, working at an acceleration voltage of 200 kV and equipped, relevant for this project, with a field emission gun and automatic cryo-box. The images were acquired in a low dose modality with a GATAN Ultrascan 1000 2k × 2k CCD.

2.8 Particle stability studies

For the evaluation of the short-term stability of LZ-SLNs produced by microfluidics, a measurement of the size of the nanosystems was performed after incubation with phosphate saline buffer (PBS, pH 7.4) and human serum (Heat Inactivated, USA origin, sterile-filtered). Briefly, 100 μL of the formulation was incubated with 1 mL of each medium at 37±0.5 °C. Size analyses were performed at fixed time points (0.5, 10, 30, 60, 90, 120 min) by diluting a certain amount of each incubated sample with water. All the experiments were performed in triplicate. For long-term stability, size measurements were conducted at 30 and 90 days after storage at 4 °C. Each sample was diluted in water. All the experiments were performed in triplicate.

2.9 In vitro release studies

Release studies of LZ from SLNs were performed using Franz diffusion cells (Arduino et al., 2020a). Experiments were conducted investigating the SLN formulation produced by the microfluidic technique. Briefly, 500 μL of LZ SLNs were placed on the diffusion barrier (area of 0.6 cm^2) made by an artificial cellulose acetate membrane (50 kDa, Fisher Scientific, Milano) which separates donor

and receptor compartments. Two release studies were conducted simultaneously. In the first, LZ SLNs were diluted with PBS (500 μL, pH 7.4), in the second with acetate buffer (500 μL, pH 4.5). PBS (pH 7.4) was used as a receptor medium for both Franz cells. The entire system was kept at a temperature of 37±0.5 °C. For each tested formulation, at predetermined time points, the same volume (300 μL) of receptor phase was collected within 168h and the same amount of fresh PBS was added in the receptor compartment to preserve sink conditions. The collected solution was analyzed by Bradford test to quantify the LZ released. Each experiment was performed in triplicate.

2.10 Enzyme activity studies

The LZ released activity studies were performed according to the procedure reported into Lysozyme Detection Kit technical bulletin (Sigma-Aldrich, RBG, RC, JJ, GCY, MAM 06/17-1). A potassium phosphate reaction buffer (50mM, pH 6.24, at 25 °C) was prepared. The suspension of *Micrococcus Lysodeikticus* (0.01% w/v) was prepared immediately before use with the reaction buffer. For the LZ control, a solution of 200 - 400 units/mg LZ was prepared in cold reaction buffer. Briefly, 800 μL of the *Micrococcus Lysodeikticus* cell suspension was pipetted into one well for the blank, one for the control, and one for each analyzed sample using a 24 multi-well plate COSTAR® 3526 (Corning Incorporated, Corning, NY 14831). 30 μL of buffer was added for the blank, 30 μL of LZ solution for the control, and 30 μL for each sample analyzed. The absorbance trend at 450 nm was evaluated using a spectrophotometer (Tecan, Infinite® 200 PRO) at a temperature of 25°C. Each experiment was performed in triplicate. The enzyme activity was expressed as Units/mL. (Unit Definition = One unit of LZ will produce a $\Delta A_{450\ nm}$ of 0.001 per minute at pH 6.24 at 25°C). Then, the Eq. (2) below was used for calculations:

$$\text{Units/ mL enzyme} = (\Delta A_{450/min}\ \text{Test}) - (\Delta A_{450/min}\ \text{Blank})\ (df) / (0.001)\ (0.1) \quad (2)$$

where *df* is the dilution factor, 0.001 is related to the change in absorbance (ΔA_{450}) as for Unit Definition, and 0.1 is related to the Volume (mL) of the enzyme solution.

2.11 Statistical analysis

All data were collected in triple replicates unless stated otherwise. Data are shown when needed as a mean value, displaying a ± standard deviation (SD) value. When comparing data sets for statistical significance, one-way ANOVA analysis was performed (Version GraphPad Prism 8.0.2), adhering to a p value of ≤ 0.05. Statistically differences are reported as follow: ns= p value > 0.05; *= p < 0.0332; **= p value < 0.0021; ***= p value < 0.0002; **** = p value < 0.0001.

3. Results and Discussion

3.1 Bench-top fabrication of Solid Lipid Nanoparticles.

The extensive experience of the authors on the bench-top production of SLNs, as documented in previous papers (Arduino et al., 2020a, 2020b; Iacobazzi et al., 2022), has allowed them to identify all the limitations in the conventional production of nanocarriers. As it is widely known, a bench-top fabrication process involves long production times, limited yielding, and modest batch-to-batch reproducibility (van Ballegooie et al., 2019). These limitations were the starting point for implementation of the microfluidic technique in the SLN production pipeline. First, it should be noted that a flow-reaction process is continuous in nature making the manufacturing process automatic and operator independent, thus reducing the source of inconsistency between batches (Webb et al., 2020). Moreover, the fine control of the independent parameters (FRR, FTR, geometry of the microfluidic mixer) facilitates the identification of optimal conditions resulting in highly homogeneous SLNs (Kimura et al., 2018). As defined above, this work aimed to be a comparative study for enzyme-loaded SLN obtained via two different fabrication approaches: a conventional bench-top method and a microfluidic-based method. Bench-top SLN were produced at increasing amounts of LZ. To obtain a proper comparison between the two processing strategies, each formulation was identified uniquely by the ratio of LZ to lipid content, being namely 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25.

By analyzing the results for the SLNs produced by the conventional bench-top method (**Table 1**), a progressive decrease in hydrodynamic particle size was observed with the LZ-to-lipid ratio. The empty SLNs returned a mean diameter of about 200 nm which jumped to 340±8.92 nm for a modest 0.025 LZ-to-lipid ratio, then reduced to 259±6.19 nm and 201±6.77 nm for LZ-to-lipid ratios of 0.05 and 0.01, respectively. At higher LZ-to-lipid ratios, the SLN hydrodynamic diameters did not change significantly falling in a small neighbor of 200 nm. This data would suggest that the LNP core is stabilized as the enzyme concentration increases, likely due to electrostatic interactions resulting from the opposite surface polarities of the cetyl palmitate molecules and the LZ enzyme that would promote the compaction of the nanostructure. This hypothesis was also confirmed by others who noted the possibility to create a complex between ammonium/ion proton and the carbon-carbon double bond of unsaturated wax esters via cation-π interaction. Moreover, it was also suggested that cetyl palmitate, which exhibits an ester function, could have a negative partial charge dispersion on the oxygen atom that could bind the protonated amino groups of the LZ, resulting in stabilization of the SLNs due to electrostatic interactions (Chen et al., 2015; Deakyne, 1985). Indeed, it was verified that an aqueous solution of LZ alone (1 mg/mL) would return a positive ζ-potential of + 9.52 ± 1.21 mV at 25°C. The LZ solution showed a positive charge because of the protonated polarized groups resulting from the 2% (w/v) surfactant solution at pH = 6.30, which is lower than the isoelectric point of the enzyme (Kuramitsu and Hamaguchi, 1980).

Regarding the surface electrostatic charge of the SLNs, a progressive increase in ζ-potential values was observed with the LZ-to-lipid ratio. Namely, from **Table 1**, the ζ-potential increased from about -15 mV for the empty SLNs all the way up to + 36 mV for a 0.25 LZ-to-lipid ratio. The strong positive charge of the SLNs at the higher LZ loading ratios would suggest that the enzyme was only partially entrapped in the core. The adsorption of LZ on the surface of the SLNs was associated with the interaction of the protein with Lutrol F68, which stabilizes the nanoparticles. Indeed, Gobbert and Müller (2005), using different types of Lutrol, confirmed the interaction of the proteins with the

surfactants and demonstrated changes in the pattern of adsorbed proteins in correlation with the type of surfactant used to produce the SLNs. It was also possible to notice an increase in encapsulation efficiency with the LZ-to-lipid ratio, obtaining EE% values ranging from 32.15% at a 0.025 LZ-to-lipid ratio, peaking at 0.15 LZ-to-lipid ratio with an EE = 53.58% and reducing down to 42.06% at 0.25 LZ-to-lipid ratio (**Table 1**).

LZ-to-lipid ratio	d_{mean} (nm)	Polydispersity index (PDI)	Z-potential (mV)	Encapsulation Efficacy %
Empty SLNs	195±4.24	0.08±0.01	- 15.2±0.65	-
0.025	****340±8.92	0.16±0.07	- 12.6±0.37	32.15±1.24
0.05	****259±6.19	0.21±0.06	+ 17.6±0.70	42.03±2.36
0.01	201±6.77	0.08±0.04	+ 24.4±0.30	43.97±1.79
0.15	*180±7.46	0.11±0.05	+ 28.8±0.07	53.58±1.13
0.20	***167±4.55	0.08±0.03	+ 31.8±1.25	44.20±3.15
0.25	197±8.40	0.08±0.04	+ 35.9±0.17	42.06±2.47

Table 1. Hydrodynamic diameter, polydispersity index (PDI), surface ζ-potential, and EE% of LZ-SLNs obtained by bench-top method as a function of the LZ-to-lipid ratio. One-way ANOVA was used to calculate statistical significance of the LZ SLNs size versus empty SLNs control [ns= p value > 0.05; *= p < 0.0332; **= p value < 0.0021; ***= p value < 0.0002; **** = p value < 0.0001].

3.2 Microfluidics-based fabrication of Solid Lipid Nanoparticles.

The bench-top production method data were then compared with those associated with the SLNs produced via the microfluidics-based approach. In a previous study, Arduino et al. (2021b) had developed *in-house* a glass microfluidic device that was suitable for SLN production. Therefore, the type of surfactant, lipid, and their relative concentrations in the aqueous and organic phases were used based on this previous work of the authors. However, in this work, a microfluidic channel with a

different geometry was realized and the effect of the total flow rate (TFR) and FRR on the LNP properties was extensively explored. The SLN production via a microfluidic technique required a fine temperature control, as cetyl palmitate is soluble in ethanol only a temperature above 50°C (D'Addio and Prud'homme, 2011). For this reason, an infrared lamp was included in the system to keep the solution warm in the injection syringes, while the microfluidic device was placed in a hot water bath at 60°C. This was sufficient to prevent the cetyl palmitate from precipitating into the microfluidic device and clog the internal channels.

Briefly, the organic phase consisting of cetyl palmitate in ethanol was pumped into the device simultaneously with the aqueous phase stabilized by the surfactant Lutrol F68 at 2% w/v. The first optimization step involved the use of different FRR between the aqueous and organic solutions. The experimental tests were performed following the idea of gradually increasing the TFR, then balancing the FRR according to the obtained results. Indeed, as reported in literature, higher TFR would favor the formation of smaller lipid nanoparticles (Arduino et al., 2021b; Roces et al., 2020). Then, each produced batch was analyzed returning the data summarized in **Table 2**. Regarding the achieved results, formulation 12 was obtained by using FRR of 5:25 mL/min for the organic and aqueous phases, respectively, and a TFR of 30 mL/min, resulting the better performing nanoformulation in terms of size (168.2±3.559 nm) and PDI (0.145±0.023).

Formulation	**Flow Rate Ratio (mL/min)**	**Total Flow Rate (mL/min)**	**d_{mean} (nm)**	**Polydispersity index (PDI)**
1	0.2:8	8.2	351.1±8.135	0.313±0.037
2	0.5:10	10.5	381.3±41.04	0.363±0.071
3	1:10	11	477.0±102.8	0.432±0.061
4	2:10	12	322.5±24.39	0.401±0.096
5	1.5:13.5	15	533.8±164.6	0.435±0.224
6	1.9:13.1	15	415.4±53.19	0.394±0.055
7	2.5:12.5	15	531.9±15.02	0.445±0.115
8	4:11	15	290.0±18.28	0.339±0.071

9	1.6:16.4	18	292.3±7.219	0.211±0.036
10	2:16	18	625.7±61.19	0.595±0.060
11	3:15	18	206.8±4.141	0.185±0.037
12	5:25	30	168.2±3.559	0.145±0.023

Table 2. Evaluation of the size and PDI of the SLNs obtained by varying the TFR and the FRR during the optimization process.

After the setting of the parameters that enabled the most successful formulations via microfluids (5:25 mL), LZ SLNs were produced. Similar to bench-top production, SLNs in microfluidics were produced by varying the LZ-to-lipid ratios in order to assess what was then the optimal concentration suitable for the *in-flow* production. Basically, the concentration ratio used between the amount of LZ placed in the aqueous phase and the lipid were kept identical to the quantities used with the bench-top method to make the study highly comparative. **Table 3** provides a summary of all resulting data. Interestingly, the same trend was found with the *in-flow* production technique involving growth of the hydrodynamic diameter of the nanoparticles in accordance with the increase of the amount of added LZ. The growth trend of the Z-potential remained unchanged from that observed by bench-top production, reflecting the hypothesized mechanism. Again, the formulation that better performed was the 0.15 LZ-to-lipid ratio, and it was used as standard for subsequent studies.

LZ-to-lipid ratio	**d_{mean} (nm)**	**Polydispersity index (PDI)**	**Z-potential (mV)**	**Encapsulation Efficacy %**
0.025	**135±2.81	0.21±0.01	- 17.2±4.15	43.20±3.18
0.05	143±3.42	0.18±0.01	- 10.6±1.03	45.96±1.17
0.01	137±3.94	0.18±0.01	+ 3.15±0.73	54.36±2.89
0.15	158±4.86	0.25±0.04	+ 9.64±0.42	70.15±1.65
0.20	***142±1.65	0.15±0.03	+ 8.85±0.69	49.58±2.64
0.25	***212±4.21	0.19±0.06	+ 17.9±0.36	57.45±2.15

Table 3. Comparison of size, polydispersity index (PDI), Z-potential, and EE% of LZ SLNs obtained by microfluidic technique. Each formulation is reported as the ratio between the amount of LZ and lipid. One-way ANOVA was used to calculate statistical significance of the LZ SLNs size versus empty SLNs control [ns= p value > 0.05; *= p < 0.0332; **= p value < 0.0021; ***= p value < 0.0002; **** = p value < 0.0001].

By comparing data resulting in **Table 1** and **Table 3**, it was demonstrated the high efficiency of *in-flow* production. In fact, the zigzag bas-relief pattern into the developed 3D printed device promoted the efficient mixing of the two solutions, as reported in the previous work after computational fluid dynamic studies (Tiboni et al., 2021). It is widely documented in the literature that the use of geometries promoting extensive mixing allows a reduction in lipid nanoparticle size (Zhigaltsev et al., 2012). For instance, Belliveau demonstrated that the use of a microfluidic device equipped with a staggered herringbone micromixer allowed the production of lipid nanoparticles in the size range 20 – 100 nm, with low polydispersity, high siRNA encapsulation efficiencies, improved scalability, and comparable or even higher gene silencing potency as compared to conventional formulation processes (Belliveau et al., 2012).

In addition, Maeki et al. (2017) studied various parameters that affect the size of lipid nanoparticles obtained by microfluidic. Also in this case, it has been highlighted that the presence of a micromixer that encourages chaotic mixing within the geometry of the device allowed to obtain nanoparticles smaller and more monodisperse. In addition, this work focused on the importance of FRR in producing smaller systems. In this study, the process of formation of lipid nanoparticles in microfluidics was described. In particular, from studies of fluid dynamics it has been underlined that when lipid nanosystems were produced there was the meeting between an aqueous phase and an organic phase, miscible with the water, containing the lipid. Therefore, the higher and faster the dilution of the organic phase into the aqueous phase was, the smaller was the size and polydispersion of the produced nanosystems (Maeki et al., 2017).

3.3 Electron microscopy characterizations of Solid Lipid Nanoparticles.

To establish the morphology and three-dimensional structure of the manufactured nanoparticles, negative staining TEM imaging analyses were carried out. These analyses were performed on both bench-top and microfluidic samples, with a focus on both empty and LZ SLNs. Resulting data are shown in **Figure 1**. By comparing the empty SLNs generated by the two manufacturing strategies, it was possible to observe how the nanosystems made using the classic bench-top method had quite irregular and not extremely spherical morphology (**Figure 1 A/B**). These SLNs would appear to have been produced by cetyl palmitate deposition, in fact the structure was found to consist of denser lipidic concentric lamellae with a core inside that had a more diffuse and deflated appearance. In contrast, the empty SLNs produced by microfluidics had spherical morphologies, which were extremely regular and reproducible (**Fig. 1 C/D**). However, these too were not particularly compact although the lipid was uniformly distributed along the surface structure of SLNs (**Figure 1 C/D**).

Concerning the LZ SLNs, produced by both manufacturing process (**Figure 2**), it was possible to notice a more compact and regular structure regarding microfluidic-based nanoparticles (**Fig. 2 C/D)** compared to bench-top ones (**Fig. 2 A/B**). Indeed, as already mentioned above for the SLNs produced by the bench-top method, also for those produced in microfluidics, it was suggested that the reduction in the dimension of the SLNs as the LZ-to-lipid ratio increased was associated with an interaction between the cetyl palmitate and the LZ and thus with greater compaction of the nanoparticle system. These morphological analyses highlighted that the SLNs containing LZ were more homogeneous and denser allowing to support the hypothesis made before in which the presence of the enzyme itself acted as a kind of trigger for the formation of a stable core for the nanoparticles. Indeed, the presence of the LZ in solution has provided an anchorage point for lipid deposition, as electrostatic interactions were formed that stabilized the nucleus of the nanosystem itself, enabling the formation of more structured nanoparticles (Chen et al., 2015; Deakyne, 1985). A peculiar morphology was noted for the SLNs which was investigated further. More specifically, Cryo-EM analyses were carried out to assess the actual structure of the SLNs. By its nature, this fine instrumental technique allowed the

water of hydration of SLNs to be retained, and thus made it possible to assess their actual nature in solution. To our knowledge, Cryo-EM analyses of SLNs produced by microfluidic were conducted for the first time. The resulting data are shown in **Figure 3**. The surface morphology of a lipid nanosystem has been illustrated in detail, and it was possible to identify the similarity between the surface of the nanosystems and the shell of a turtle, and in fact the term turtle-SLNs was coined. The turtle-like shape has been clearly identified in empty and loaded nanosystems produced by bench-top method (**Fig.3A/B**), so it is likely that this has always been the morphology of SLNs. The specificity of the turtle-like structure has probably been the result of the nanosystems formation. The manufacturing process of LZ SLNs has provided a meeting point between cetyl palmitate in the organic phase and LZ in the aqueous phase. As defined above, the cetyl palmitate showed a partially charge dispersion on the oxygen of the carbonylic group thus generating an electrostatic interaction with the protonated ionizable groups of the LZ and stabilizing the lipidic core (Chen et al., 2015; Deakyne, 1985). For empty SLNs the stabilizing process has been due to the presence of surfactant in the aqueous phase (Göppert and Müller, 2005). Then, the rapid dispersion of the organic phase within the aqueous phase has stopped the excessive growing of the lipid nuclei formed by nanoprecipitation (Maeki et al., 2017), and the smallest semi-stable nuclei have likely decorated the surface of the bigger ones. This resulted into one monodisperse population of SLNs characterized by turtle-like morphology.

(INSERT FIGURE 1)

Figure 1. Representative TEM micrographs obtained by negative staining of SLNs. (A/B) Micrographs of empty SLNs produced by bench-top method. (C/D) Micrographs of empty SLNs produced by microfluidic technique.

(INSERT FIGURE 2)

Figure 2. Representative TEM micrographs obtained by negative staining of LZ SLNs. (A/B) Micrographs of LZ SLNs produced by bench-top method. (C/D) Micrographs of LZ SLNs produced by microfluidic technique.

(INSERT FIGURE 3)

Figure 3. Representative CRYO-EM micrographs of the turtle-like SLNs. (A) Micrographs of empty SLNs produced by bench-top method. (B) Micrographs of LZ SLNs produced by bench-top method. (C) Micrographs of empty SLNs produced by microfluid technique. (D) Micrographs of LZ SLNs produced by microfluidic technique.

3.4 Stability studies

In literature have been found examples of studies in which the stability of these colloidal nanosystems has been investigated (Arduino et al., 2021b). To our knowledge, there is no information concerning the colloidal stability of biologically active molecules loaded SLNs produced via microfluidic technique. Therefore, studies assessing the stability were carried out of both empty and LZ SLNs (LZ-to-lipid ratio 0.15) produced by microfluidics.

Size measurements were conducted over 2h at set time points incubating the formulation at 37.0 ± 0.5 °C, using PBS (pH 7.4) and diluted 1:5 human serum, as relevant media (**Figure 4**). The behavior of LZ SLNs revealed a stable profile while investigated in PBS (**Figure 4A**). It was evident that for the LZ SLNs in PBS (**Fig. 4A**), there is no significance in the size variation over time (p value ns). This trend might be due to the presence of LZ, that has partly adsorbed on the surface of the nanoparticles generated a dynamic equilibrium of adsorption-desorption from the lipid phase contributing to the light fluctuating size trend (**Fig. 4A**). As hypothesized before (paragraph 3.3), LZ acted as stabilizing element of SLNs contributing to the formation of more stable structures compared

to empty ones (Chen et al., 2015; Deakyne, 1985). Thus, this evidence reflected on PBS colloidal stability, in which LZ SLNs resulted more stable over time compared to empty SLNs which showed more variability in size and PDI (**Fig. 4 A/C**).

On the other hand, regarding colloidal stability in human serum (**Figure 4B**), it was noted a more remarkable growing trend for LZ SLNs. Statistical analysis revealed a significant difference in term of size related to the complexity of the investigated medium. To explain this trend, it should be necessary to take into account the protein-corona effect (Mishra et al., 2021). The formation of protein-corona is a phenomenon that occurs when a nanosystem encounters a biological fluid. This state results in nanosystems surface-enrichment of substances, mostly proteins, causing important changes in size, properties, and surface charge (Wang et al., 2021; Zanganeh et al., 2016). Systemic studies on the interaction between proteins and SLNs once introduced into the systemic circulation are still missing (Wang et al., 2021), therefore an assumption has been made here considering the behavior of LZ SLNs in human serum. Typically, protein-corona binding happens in an extremely dynamic process along with continuously adsorbing and desorbing of protein molecules (Forest and Pourchez, 2017). Analyzing data about LZ SLNs in human serum, the upward trend was justified as a result of the dynamic adsorption-desorption phenomena of both LZ and serum proteins on the surface of SLNs.

Empty SLNs in human serum showed no significance about size and PDI (**Fig. 4 B/D**).

(INSERT FIGURE 4)

Figure 4. Stability studies at 37 °C in relevant media for SLNs produced by microfluidics. Hydrodynamic diameter in: (A) empty SLNs and LZ SLNs (LZ-to-lipid ratio 0.15) in PBS (pH 7.4), (B) empty SLNs and LZ SLNs (LZ-to-lipid ratio 0.15) in human serum. PDI in: (C) empty SLNs and LZ SLNs (LZ-to-lipid ratio 0.15) in PBS (pH 7.4), (D) empty SLNs and LZ SLNs (LZ-to-lipid ratio 0.15) in human serum. One-way ANOVA was used to calculate statistical significance of the empty SLNs and LZ SLNs size and PDI versus empty SLNs

and LZ SLNs control (t= 0 min) in the same medium [ns= p value > 0.05; *= p < 0.0332; **= p value < 0.0021; ***= p value < 0.0002; **** = p value < 0.0001].

Long-term stability of LZ SLNs (LZ-to-lipid ratio 0.15) produced via microfluidic technique showed little change in sample size during storage (0, 30 and 90 days) at 4 °C, probably due to the above-mentioned adsorption-desorption equilibrium performed by LZ (**Figure 5**).

(INSERT FIGURE 5)

Figure 5. LZ SLNs long-term stability at 4°C. Size and PDI measurements were conducted at fixed time points (0, 30, 90 days) after the microfluidic-based production. One-way ANOVA was used to calculate statistical significance of the LZ SLNs size and PDI versus LZ SLNs control at day 0 [ns= p value > 0.05; *= p < 0.0332; **= p value < 0.0021; ***= p value < 0.0002; **** = p value < 0.0001].

3.5 In vitro release study and enzymatic activity test

The *in vitro* release study was conducted as reported in literature (Arduino et al., 2020a, 2021a). A membrane (Spectra/Por®6 Dialysis Membrane, MWCO: 50 kDa) was used as separating barrier between the donor and acceptor compartment to let the LZ pass through once released by the SLNs. In our study, complex media as serum or plasma were not used and it was not added any lytic enzymes to the formulation in the donor compartment to avoid interference with the LZ quantification test. The experiments were carried out to assess the release kinetics in buffered solutions at pH 7.4 and pH 4.5. These two pH values were considered biologically relevant to study the response of the formulation in biological condition to mimic blood flow and endosomal microenvironment once administered via parenteral route (Duskey et al., 2020; Patel et al., 2019). Resulting data have been shown in **Figure 6**. As expected in absence of plasmatic degradants, with only PBS, LZ-SLNs have performed an initial burst effect in the first 4 hours of incubation, subsequently demonstrating a sustained release over time up to 67 % of the total load at 168 hours (7 days) (Èller et al., n.d.; Wissing

et al., 2004). In presence of acetate buffer in the donor compartment, the burst effect was more remarkable and continuous up to 68% of the load in the first 24 hours, followed by a sustained release up to 80% at 168 hours (7 days). From these results, SLNs had a short period of burst release followed by a sustained and slow release. In many cases, the burst release is due to those hydrophilic peptides and proteins accumulated at the o/w interface and in the outer shell during preparation (Almeida and Souto, 2007; Èller et al., 2000), which is mainly a combination of desorption and diffusion processes. The prolonged slow release owes to the protein incorporated into the particle core, which mainly depends on diffusion process *in vitro* (Xie et al., 2008). Because the lipid matrix could not be digested in vitro, the incorporated protein may remain in the particle core. However, *in vivo* release could be much faster because of the degradation of the lipid matrix caused by enzymatic digestion. Furthermore, contrasting what might be explored *in vitro*, in a more physiological condition it should be considered the protein-corona effect. From data reported in literature, it has shown that the burst effect could be significatively reduced by the binding of proteins on the surface of nanosystems, leading a change in the entire release profile (Zanganeh et al., 2016).

Interestingly, although cetyl palmitate was found to be stable in a wide range of pH values (Zimmermann and Èller, 2001), the profile of the same formulation in presence of acetate buffer (pH 4.5) in the donor compartment returned a faster and appreciable release even in absence of lytic degradants. This phenomenon could be explained considering the increased solubility of LZ at pH 4.5 compared to pH 7.4 (Holland et al., 1991). As reported in literature, it has been fundamental to consider the pH of the dissolution medium in the case of ionizable therapeutic molecules, as the increased solubility of a drug in a relevant medium led to an improved release profile (Madan et al., 2013).

Both profiles could be explained considering the intimate nature of SLNs as drug delivery system (Wissing et al., 2004). Indeed, the almost immediate fast release of LZ was due to the presence of enzyme adsorbed on the surface of the nanosystems. Using exclusively PBS the situation of the nanoparticle circulating in the blood stream was mimicked in an extremely simplified manner,

showing the great protection that the solid lipid core could provide. On the other hand, the addition of a small amount of buffered solution at lower pH has simulated the biological environment after endocytosis and endosome formation (Duskey et al., 2020). This endosome-like condition has proven that SLNs could allow a magnified release of the cargo once arrived at the target tissue and after the cellular internalization, avoiding non-specific release.

(INSERT FIGURE 6)

Figure 6. Release profiles of LZ SLNs (enzyme/lipid ratio 0.15) in PBS (pH 7.4). The release profile performed by adding buffered solution at pH 4.5 is underlined in red, while the release profile performed by adding buffered solution at pH 7.4 is underlined in blue.

Subsequently, the *in vitro* release samples in PBS at fixed time points (24h, 72h, 168h) were used to perform the LZ activity assay on *Micrococcus Lysodeikticus* suspensions.

To evaluate the assay more specifically, activity tests were performed even on empty SLNs. The resulting data demonstrated that the release from empty SLNs did not induce lytic effect with respect to the used substrate. This evidence confirmed that the detected biological activity had to be ascribed to the enzyme and not to the effect of nanosystems constituting material. As it has been shown in **Figure 7**, the LZ activity has been reported as U mL^{-1} enzyme applying Eq. (2) (al Meslmani et al., 2016; Cerón et al., 2021).

As expected from previous experiments, the increase in the lytic effect on the substrate was due to the major amount of released LZ over time (32 U mL^{-1} at 24h, 173 U mL^{-1} at 72h, 460 U mL^{-1} at 168h). The activity assay that was performed using samples deriving from release study with buffered solution at pH 4.5 in the donor compartment was not reported here as showed no significant differences from the pH 7.4.

Thus, the obtained data have suggested that the condition used during the manufacturing process by microfluidics did not affect the LZ ability to perform its biological activity once released.

(INSERT FIGURE 7)

Figure 7. LZ activity of control, release from empty SLNs, release from LZ SLNs at fixed time points (24h, 72h, 168h) on *Micrococcus Lysodeikticus* suspensions.

4. Conclusion

Using biological molecules as therapeutic agents is one of the most fascinating and intriguing frontiers in pharmaceutical technology. Unfortunately, their limited chemical-physical stability upon direct administration has hampered their broad application. In this context, nanoscale drug delivery systems could be key to improve encapsulation, protect the therapeutic agent, and optimize delivery to the biological target. The microfluidic technique has proven to be one of the most promising methodologies to scale-up the production of nanocarriers.

On this basis, in this exploratory study a systematic approach used for the production and analysis of SLNs carrying LZ, a model enzyme, was described. By comparing the classical bench-top method with the microfluidic technique, the *in-flow* manufacturing process of nanosystems was optimized. For the first time it was exploited the use of polypropylene 3D printed device to obtain LZ SLNs. Moreover, the detailed methodological study performed on SLNs produced by microfluidics allowed to obtain important structural information regarding the intimate morphology of these lipid nanovectors. Data from negative staining TEM and Cryo-EM analyses returned a peculiar surface architecture that was named “turtle-like”. At authors’ knowledge, this was never reported before. Furthermore, the performed *in vitro* release studies highlighted the ability of LZ SLNs to realize a prolonged release over time. The post-release enzymatic activity studies have showed that the LZ

maintained its activity on biological substrates, thus proving that the production process did not affect its intrinsic activity. This evidence has been crucial in identifying microfluidic technique as the most promising and feasible way to successfully introduce biologicals in therapy.

CRediT authorship contribution statement:

Federica Sommonte: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Ilaria Arduino**: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Rosa Maria Iacobazzi**: Methodology, Validation. **Mattia Tiboni**: Resources, Investigation. **Federico Catalano**: Formal analysis, Data curation. **Roberto Marotta**: Formal analysis, Data curation. **Martina Di Francesco**: Investigation. **Luca Casettari**: Resources, Investigation, Review & editing. **Paolo Decuzzi**: Methodology, Data curation, Review & editing. **Angela Assunta Lopedota**: Validation, Data curation. **Nunzio Denora**: Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements:

Nunzio Denora acknowledges the University of Bari "Aldo Moro" (Italy) for their financial and technical support.

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