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Design and physicochemical characterization of novel hybrid SLN-liposome nanocarriers for the smart co-delivery of two antitubercular drugs

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Abstract:	In the present work a novel hybrid system for the delivery of two first-line antitubercular drugs, rifampicin (RIF) and isoniazid (INH), was designed. In order to control the release of the drugs and improve the efficiency of conventional carriers, like liposomes or solid lipid nanoparticles (SLNs), the new systems were developed by embedding SLNs into lecithin-based liposomes through the reverse-phase evaporation method. The hybrid system was characterized and compared to SLNs and liposomes in terms of size, encapsulation efficiency, morphology, and drug release. Detailed structural data and further evidence of the successful formation of the hybrid nanoparticles were obtained by applying small-angle neutron scattering (SANS). The hybrid system displayed a particle size comparable to liposomes and a high encapsulation efficiency. Morphological results obtained by atomic force microscopy (AFM) highlighted the possible presence of SLNs into the phospholipid bilayer; this hypothesis was supported by the slower in vitro release of the hydrophilic drug INH compared to liposomes and SLNs. Moreover, scattering differences of the inner core of the nanoparticles, evidenced in the SANS analysis, further corroborated the successful formation of the hybrid carrier. These novel systems were able to release their content as expected from an efficient dosage form in a perspective of an inhaled administration, improving the stability and the drug release profile with respect to plain liposomes. The physicochemical characterization of our systems opens new avenues towards a better understanding of the formulation of vesicles encapsulating SLNs.
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Journal of Drug Delivery Science and Technology

Dear Editor,

We would greatly appreciate if you could consider the manuscript entitled "Design and physicochemical characterization of novel hybrid SLN-liposome nanocarriers for the smart co-delivery of two antitubercular drugs" by *Eleonora Truzzi, Angela Capocefalo, Fiorella Meneghetti, Eleonora Maretti, Matteo Mori, Valentina Iannuccelli, Fabio Domenici, Carlo Castellano and Eliana Leo* for publication in the *Drug Delivery and Translational Research* journal.

We report on the formulation and characterization of new core-shell SLN/liposome carriers designed to better control the release of drugs (two first-line antitubercular drugs) with respect to conventional liposomes or solid lipid nanoparticles, which suffer from known issues, such as the leakage of small hydrophilic drugs or the low loading capacity. The small-angle neutron scattering analysis allowed us to precisely define the variation in size of the nanoparticles after the encapsulation of co-loaded SLNs. The deep physicochemical characterization of our systems may open new avenues towards a better understanding of the formulation of vesicles encapsulating SLNs. These novel hybrid systems offer various benefits with respect to conventional nanocarriers, such as a higher encapsulation efficiency, controlled particle size, and the capacity to load multiple therapeutic agents, inducing a prolonged drug release.

We are confident that this article might be of interest to a quite broad readership, and we hope that the quality of the presented data meets the standards of *Journal of Drug Delivery Science and Technology*.

We would like to underline that this manuscript is not under consideration for publication in any other journal and has not been published elsewhere.

Thank you in advance for the attention you will pay to the manuscript.

On behalf of the other corresponding authors Carlo Castellano and Fabio Domenici

Yours sincerely,

Eliana Leo

Modena (Italy), October 6th, 2021

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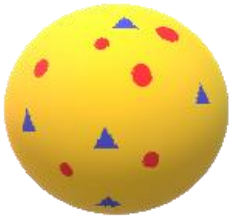
Graphical Abstract

Formulation

Solid lipid nanoparticles (SLN)



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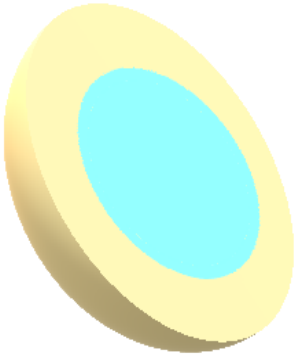
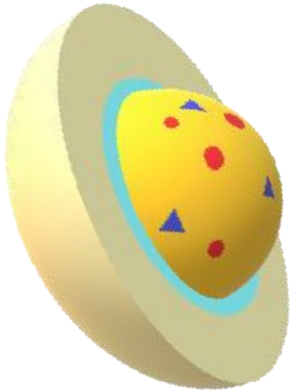


Rifampicin

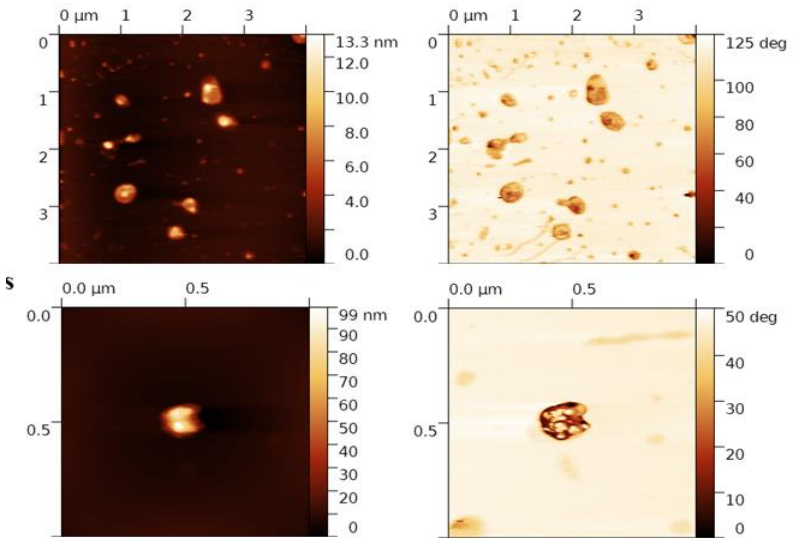
Isoniazid

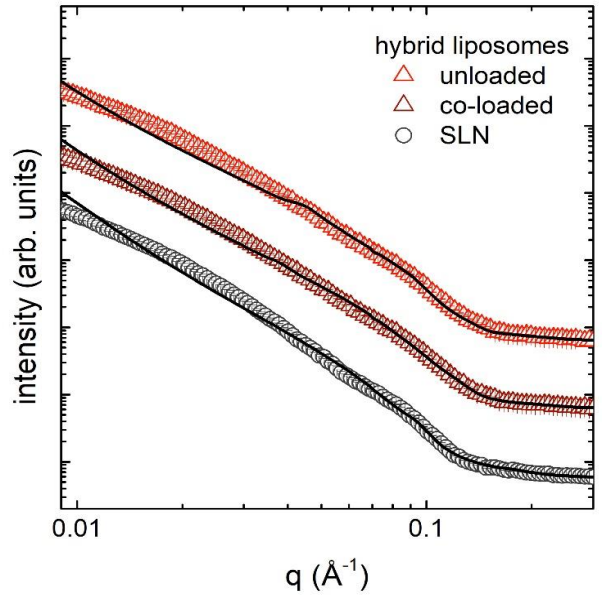
Hybrid SLN-liposomes

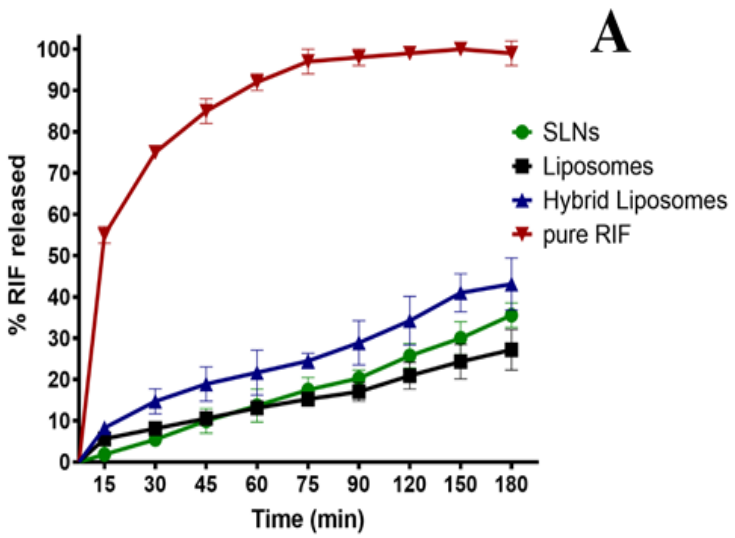


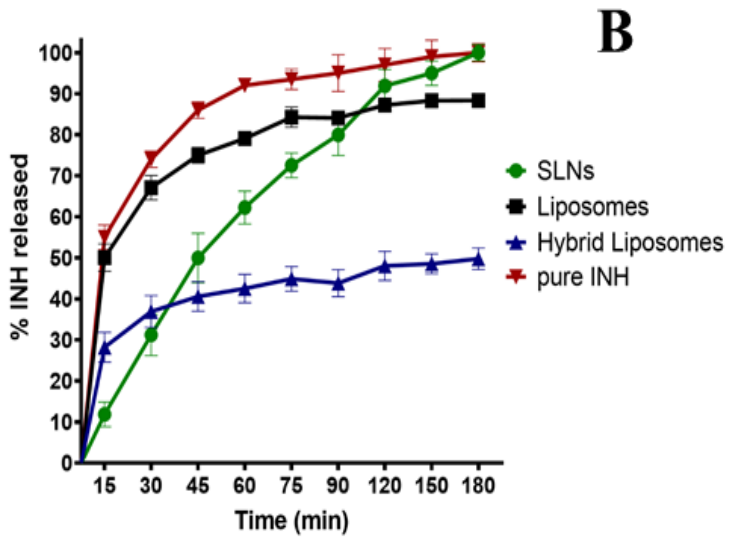
Characterization

Hybrid SLN-liposomes









Design and physicochemical characterization of novel hybrid SLN-liposome nanocarriers for the smart co-delivery of two antitubercular drugs

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Abstract

In the present work a novel hybrid system for the delivery of two first-line antitubercular drugs, rifampicin (RIF) and isoniazid (INH), was designed. In order to control the release of the drugs and improve the efficiency of conventional carriers, like liposomes or solid lipid nanoparticles (SLNs), the new systems were developed by embedding SLNs into lecithin-based liposomes through the reverse-phase evaporation method. The hybrid system was characterized and compared to SLNs and liposomes in terms of size, encapsulation efficiency, morphology, and drug release. Detailed structural data and further evidence of the successful formation of the hybrid nanoparticles were obtained by applying small-angle neutron scattering (SANS). The hybrid system displayed a particle size comparable to liposomes and a high encapsulation efficiency. Morphological results obtained by atomic force microscopy (AFM) highlighted the possible presence of SLNs into the phospholipid bilayer; this hypothesis was supported by the slower *in vitro* release of the hydrophilic drug INH compared to liposomes and SLNs. Moreover, scattering differences of the inner core of the nanoparticles, evidenced in the SANS analysis, further corroborated the successful formation of the hybrid carrier. These novel systems were able to release their content as expected from an efficient dosage form in a perspective of an inhaled administration, improving the stability and the drug release profile with respect to plain liposomes. The physicochemical characterization of our systems opens new avenues towards a better understanding of the formulation of vesicles encapsulating SLNs.

Keywords:

SANS, AFM, Nanoparticles, Rifampicin, Isoniazid

1. INTRODUCTION

The treatment of lung infections represents one of the great challenges of our time. These conditions are caused by a variety of bacterial, viral, and fungal agents, including *Mycobacterium tuberculosis* and, more recently, SARS-COV2. Tuberculosis (TB) still ranks as the first cause of death from a single pathogen, and the Covid-19 pandemic threatens to reverse the progress made in the last years in terms of diagnosis and disease control.

Pulmonary drug delivery of nanoparticle-based therapeutics have emerged as promising strategy, allowing the improvement of the pharmacokinetic profile and therapeutic efficiency of drugs, compared to conventional delivery systems [1,2]. In this context, inhaled therapy by lipid nanoparticles offers great benefits [3]. Solid lipid nanoparticles (SLNs) are monophasic structures formed by biocompatible lipids and a hydrophilic surfactant layer that keeps them in suspension. The main advantage of SLNs is their excellent physicochemical stability, which provides greater protection against the degradation of labile drugs [4]. In the last decade, SLNs have been demonstrated to be an effective drug delivery system for anti-TB inhaled therapy [5–8]. Despite their advantages, SLNs are characterized by a low drug loading capacity and rapid release, especially when hydrophilic drugs are concerned.

Liposomes are spherical vesicles, formed by an aqueous core surrounded by bilayers of phospholipids, which can be exploited to transport both hydrophilic and lipophilic drugs through the respiratory tract [9]. Due to their high biocompatibility and easy aerosolization, liposomes represent one of the most promising drug delivery systems for the treatment of pulmonary infections [10,11].

Hybrid particles, composed by SLNs embedded in liposomes, were designed to obtain a new multi-compartmental drug delivery system. In detail, the lipid bilayer of liposomes was predicted to increase the colloidal stability and efficiency of intracellular delivery of the resulting hybrid system, while the nanoparticulate cores were employed to improve the pharmaceutical performance of liposomes. Moreover, the use of no additional chemical components allowed to avoid the risk of toxicity.

Rifampicin (RIF) and isoniazid (INH) are two first-line anti-TB agents, which are part of the standard treatment regimen for drug-susceptible TB. Recently, their co-administration (Rifinah®) has been shown to improve the clinical outcomes of TB patients [12]; in this context, the use of nanocarriers could enhance the pharmacokinetic properties of this combination, ensuring greater efficacy [13].

Our preliminary results showed that SLNs were unable to achieve an effective, durable encapsulation of the two drugs (INH, LogP -0.64; RIF, LogP 3.719) [14,15]. In particular, INH, owing to its hydrophilic nature, was rapidly released from the lipid matrix. Therefore, hybrid systems, generated by using liposomes as templates for the deposition of SLNs, were developed for the co-delivery of the two antitubercular drugs.

First, to formulate the hybrid nanocarriers we optimized the composition and the experimental parameters in preliminary studies. Then, because the successful implementation of a particle-based delivery system requires a detailed understanding of the morphology and properties of the drug carrier, the newly developed hybrid SLN-liposomes were characterized by Photon Correlation Spectroscopy (PCS), atomic force microscopy (AFM), and small-angle neutron scattering (SANS). In our previous work, we demonstrated that SANS is a powerful tool for the investigation of amphiphilic aggregates, such as liposomes [9]. SANS experimental data allowed us to characterize our nanoparticles in terms of overall size, dimension of the aqueous core and thickness of the lipid shell Moreover, it was useful to obtain indications on the localization of the drugs in the nanoparticles, through a comparison between loaded and unloaded systems within the same carrier category.
Finally, *in vitro* drug release studies were performed to gather key information on the newly developed nanoparticles in terms of effectiveness of the drug release profile.
Our results will aid researchers in the selection of the appropriate carrier based on the drug chosen in therapy, and in addressing the remaining challenges that need to be overcome to enhance the efficiency of current pulmonary delivery systems.

2. MATERIALS AND METHODS

2.1 Materials

Concerning the preparation of SLNs, Compritol ATO 888 (mono-, di- and triglycerides of Behenic acid) was a kind gift from Gattefossè (Saint Priest, Cedex, France), and Tween 80 was purchased from Sigma-Aldrich (Milano, Italy). For the preparation of liposomes, Cholesterol 95% was acquired from Acros (Geel, Belgium) and soy Lecithin from Farmalabor (Canosa di Puglia, Italy). Rifampicin (RIF) was a kind gift from Sanofi (Brindisi, Italy), while Isoniazid (INH) was purchased from T.C.I. Europe (Zwijndrecht, Belgium); these compounds were used as model drugs for all the formulations. MilliQ water was obtained from a Millipore system (Bedford, MA, USA). Deuterated 1,3-distearoyl-2-oleoyl glycerol was purchased from Toronto Research Chemicals (Toronto, Canada), and D_2O from VWR (Milan, Italy).
All employed solvents were of analytical grade.

2.2 Nanocarrier preparation

2.2.1 SLNs preparation

SLNs were prepared by the melt-emulsification technique. Briefly, the lipid phase containing 60 mg of Compritol (with 15 mg of INH and 15 mg of RIF in co-loaded particles) was melted at 85 °C and then emulsified in 3 mL of Tween 80 aqueous solution (1.7 % w/v), kept at the same temperature, by ultrasonication at 20 W (Vibra-Cell, Sonics&Materials, NewTown, CT, USA) for 1 min, followed by homogenization by Ultra-Turrax (Ika-euroturrax T 25 basic, IkaLabortechnik, Staufen, Germany) at 24,000 rpm for 1 min (T-25 basic, Ika Labortecnik, Germany). Finally, the O/W emulsion was further ultrasonicated in the same conditions for 1 min. The obtained emulsion was cooled in an ice-bath and purified 3 times by centrifugation at 6,000 x g for 30 min (Rotina 380R, Hettich, Germany) in 100 kDa MWCO Vivaspin columns (Sartorius, Goettingen, Germany). The concentrated samples were diluted with deionized water to 3 mL and used for the preparation of SLN-loaded liposomes.
For the characterization of plain SLNs, samples were freeze-dried for 24 h (Lio 5P, Cinquepascal, Milan, Italy) and then used for *in vitro* studies.
For the SANS analysis, deuterated 1,3-distearoyl-2-oleoyl glycerol (2 mg) was used in combination with Compritol.

2.2.2 Liposome preparation

Liposomes were prepared by Reverse Phase Evaporation (REV), as previously described [9]. Cholesterol (Chol) and soy lecithin at fixed molar ratio (1:1) (and 15 mg RIF for co-loaded liposomes) were solubilized in chloroform at the final concentration of 40 mM. The obtained solution was placed into a round-bottom flask, and the solvent was removed under vacuum at room temperature until the formation of a dry film (Buchi HB-140, Buchi, Switzerland). The phospholipid film was re-dissolved in diethyl ether and mixed with 3 mL of water (containing 15 mg of INH for co-loaded samples), with a ratio of ether to water of 3:1. The suspension was mixed by vortex for 3 min to form a W/O emulsion. The obtained emulsion was stirred for 2 h at 200 rpm to remove the organic solvent, thus inducing the phase reversal and, finally, the formation of liposomes. Liposomes were homogenized by Ultraturrax (Ika-euroturrax T 25) for 3 min and purified by dialysis for 30 min to separate the free drugs, before being stored at 4 °C in vials.
For the SANS analysis, liposomes were prepared and purified using D_2O instead of milliQ water.

2.2.3 Hybrid SLN-liposome preparation

Hybrid SLN-liposomes were prepared following the procedure previously described for liposomes, by using 3 mL of unloaded or co-loaded SLN suspension instead of water for the preparation of the W/O emulsion with diethyl ether. The obtained hybrid SLN-liposomes were purified by dialysis for 30 min and then stored at 4 °C. For the SANS analysis, hybrid SLN-liposomes were prepared with SLN composed by deuterated 1,3-distearoyl-2-oleoyl glycerol (2 mg), and D_2O instead of milliQ water.

2.3 Particle size and Z-potential

Particle size (average hydrodynamic diameter by intensity with polydispersity index, PDI) and Z-potential were determined by photon correlation spectroscopy (PCS), using a Zetasizer Nano ZS analyzer system (Zetasizer version 6.12; Malvern Instruments, Worcs, U.K.). The results were expressed as the average of three different measurements. Analyses were performed in triplicate and each measurement was averaged over at least 12 runs.

2.4 Atomic force microscopy (AFM)

The morphology of SLNs, liposomes, and hybrid SLN-liposomes was determined by atomic force microscopy (AFM). AFM observation was performed by Multimode with a Nanoscope 3D controller (Bruker, Karlsruhe, Germany). Topographic and phase imaging were acquired at room temperature (20 °C) and atmospheric pressure (760 mmHg), operating in non-contact mode. Immediately before the analysis, freshly prepared unloaded samples (SLNs, liposomes, and hybrid particles) were diluted in water (1:100 v/v) and deposited onto a small mica disk with a diameter of 1 cm. After 3 min, the excess of water was removed and the final images were obtained by processing the topographic and phase images with the Gwyddion (2.5 version) software.

2.5 Drug loading and encapsulation efficiency

Drug loading (DL%) and encapsulation efficiency (EE%) of loaded SLNs, liposomes, and hybrid SLN-liposomes were evaluated by UV–visible spectroscopy (Lambda 3B Perkin-Elmer, Waltham, MA, USA). The absorbance of the solutions containing the drugs was converted to the amount of drug by preparing standard calibration curves, constructed using the supernatants of the corresponding unloaded samples (n = 6), to eliminate any possible interference in the measurements. The direct quantification of RIF and INH in co-loaded nanocarriers using UV–visible spectroscopy could not be performed, due to an overlapping of the absorption spectrum of INH to that of RIF [14].

Therefore, UV spectroscopy was employed to determine INH peak-to-peak first-order derivative. The reliability of derivative UV spectrophotometry for the concurrent estimation of RIF and INH was demonstrated by Gürsoy *et al.* [15].

DL% and EE% were calculated by using the following equations:

$$DL\% = \frac{incorporated\ drug\ (mg)}{total\ mass\ of\ \ sample*\ (mg)} \times\ 100$$

$$EE\% = \frac{incorporated\ drug\ (mg)}{initial\ drug\ (mg)}\ x\ 100$$

**weighed after freeze-drying process*

2.5.1 SLN drug loading determination

For the determination of the content of the hydrophilic drug (INH) in SLNs, 10 mg of freeze-dried SLNs were dissolved in 1 mL of chloroform, and then 5 mL of MilliQ water were gradually added in portions of 1 mL, mixing by vortex for 1 min after each addition, to extract the drug. The mixture was centrifuged (Rotina 380R, Hetting, Germany) for 30 min at 9,500 x g to separate the two phases, and the aqueous supernatant containing the hydrophilic drug was analyzed using a UV-visible spectrophotometer at 262 nm (Lambda 3B Perkin-Elmer, Waltham, MA, USA).
For the determination of the hydrophobic drug (RIF), 10 mg of freeze-dried SLNs were dissolved in 1 mL of chloroform and then 4 mL of methanol were added. The obtained solution was mixed by vortex for 2 min. After centrifugation (10 min at 9,500 g) to separate potential undissolved lipids, RIF was quantified in the solution by spectrophotometry, recording the absorbance at 475 nm.

2.5.2 Liposome and hybrid SLN-liposome drug loading determination

For the determination of the hydrophilic drug (INH), 300 μL of liposomal suspension was dissolved in 1.5 mL of isopropanol, and then an aliquot of MilliQ water (5.2 mL) was added. For the determination of the hydrophobic drug (RIF), 300 μL of liposomal suspension were dissolved in 1.5 mL of isopropanol, and then 5.2 mL of methanol were added. In both cases, the drug loading was determined spectrophotometrically in the respective solution, as described in paragraph 2.5.
As for hybrid SLN-liposomes, the same procedure used for plain liposomes was adopted with a slight modification: after the addition of isopropanol, the mixture was heated at 85 °C for 10 min to allow the complete melting of the SLNs before the extraction of the drugs by water or by methanol for INH and RIF, respectively. The quantification was performed as previously described in section 2.5.

2.6 SANS analysis

SANS experiments were carried out on the time-of-flight instrument V16 at the Helmholtz-Zentrum Berlin (HZB, Berlin, Germany). A scattering vector q = (4π/λ) sinθ (where λ is the neutron wavelength and 2θ is the scattering angle) range between 0.0035 $Å^{-1}$ and 0.07 $Å^{-1}$ was obtained by using neutron wavelengths spanning from 2 Å to 9 Å, with a sample-detector distance of 2 m and 11 m, respectively. The samples were analyzed in 2 mm path length, UV-spectrophotometer-grade, quartz cuvettes and mounted on an enclosed, computer-controlled, sample chamber. All scattering data were normalized for the sample transmission, background-corrected using a quartz cell filled with the solvent (D_2O), and corrected for the linearity and efficiency of the detector response (component of the instrumental smearing). Data reduction was performed with the software package MantidPlot [16].

Experimental data were analysed using the core-shell spherical model of the fitting routine SASView 5.0.3, in the context of a spherical core-shell morphology for both liposomes and SLNs. The employed model provides the form factor *P(q)* for non-interacting spherical particles with a core-shell structure [17]:

$$P(q) = \frac{x}{V_p}\left[3V_c(\rho_c - \rho_s)\frac{\sin qr_c - qr_c\cos qr_c}{(qr_c)^3} + 3V_r(\rho_s - \rho_0)\frac{\sin qr_p - qr_p\cos qr_p}{(qr_p)^3}\right]^2$$

where x is a scale factor, V_p is the volume of the core-shell sphere with radius r_p, V_C is the volume of the particle's core, r_c is the radius of the core, ρ_c and ρ_s are the scattering length densities (SLDs) of the core and of the shell, respectively, and ρ_0 that of the solvent. The thickness t of the shell is obtained by the difference $t = r_p - r_c$.

2.7 In vitro drug release studies

The *in vitro* release of RIF and INH from SLNs, liposomes, and hybrid SLN-liposomes was determined in Simulated Lung Fluid (SLF) at pH 7.4 [18]. A specific amount of sample (45 mg of freeze-dried SLNs suspended in 1 mL of deionized water or 1 mL of liposomal dispersions) was placed into a dialysis membrane (Dialysis Tubing – Visking MWCO-12-14000 Daltons, Medicell International Ltd, London). The dialysis tube was immersed into a vessel containing 30 mL of SLF medium and maintained at 37 ± 0.5 °C under gentle stirring. At fixed time intervals, aliquots (1 mL) were withdrawn from the solution, and INH and/or RIF content was determined spectrophotometrically. Two aliquots were analyzed for each time point using unloaded carriers as

blank; the study was performed in triplicate. INH and RIF were quantified by spectrophotometry, as previously described (section 2.5).

2.8 Statistical analysis

Statistical comparison of drug content was performed by one-way Analysis of Variance (ANOVA) test, followed by Tukey's test. Differences between groups were considered to be statistically significant at $p < 0.05$.

3. RESULTS

3.1 SLNs, liposomes and hybrid SLN-liposomes characterization

In this study, a hybrid system was designed to obtain a novel nanocarrier offering an improved encapsulation efficiency and controlled release of the embedded drugs.

Size, polydispersity index (PDI), and Z-potential, determined by PCS analysis, as well as encapsulation efficiency (EE%) and drug loading (DL% w/w) of the analyzed samples are summarized in Table 1.

SLNs had a small size (about 130 nm), with a homogenous size distribution (PDI < 0.3), while liposomes were bigger (300 nm) and non-homogenous in size, with a higher PDI value, indicating a multimodal particle distribution. No significant differences in size could be appreciated between liposomes and hybrid SLN-liposomes, neither for the unloaded nor for the co-loaded samples. The size homogeneity of liposomes seemed to decrease with the incorporation of SLNs into the liposomal bilayer, considering that the PDI for hybrid SLN-liposomes was > 0.4. With respect to the Z-potential, which describes the surface particle charge, the values for all the co-loaded samples were considerably more negative compared to those of the unloaded samples.

Hence, we can conclude that we successfully obtained nanoscale devices, with a particle size within the suitable range for pulmonary delivery.

The main rationale behind the design of this hybrid carrier was to improve the encapsulation and/or release kinetic of hydrosoluble drugs. The EE% for the lipophilic drug RIF in conventional SLNs and liposomes was comparable, while for the hydrophilic drug INH the EE% were significantly lower in the case of SLNs. Despite hybrid SLN-liposomes were prepared using co-loaded SLNs, the EE% of the hybrid system revealed that no drug loss occurred during the formulation, except for a tiny leakage of RIF (about 9%), probably due to the presence of ether during the preparation of the emulsion.

Table 1. Size, PDI, Z-potential, EE% and DL% of SLN, liposomes and hybrid SLN-liposomes.

Formulation	Size (nm)	PDI	Z-potential (mV)	RIF		INH	
				EE %	DL% (w/w)	EE %	DL% (w/w)
Unloaded SLNs	122 ± 7	0.23 ± 0.01	-5.1 ± 11.9	/	/	/	/
Co-loaded plain SLNs	135 ± 9	0.25 ± 0.05	-25.3 ± 4.3	61.7 ± 3.9	10.3 ± 0.7$^{\#}$	31.3 ± 0.6$^{@}$	5.2 ± 0.1§
Unloaded liposomes	300 ± 8	0.33 ± 0.02	-40.8 ± 5.4	/	/	/	/
Co-loaded plain liposomes	310 ± 7	0.30 ± 0.05	-51.5 ± 5.4	62.1 ± 4.9	8.1 ± 0.7$^{\#}$	46.9 ± 6.5$^{@}$	6.1 ± 0.8§
Unloaded hybrid SLN-liposomes	343 ± 6	0.49 ± 0.04	-39.7 ± 5.2	/	/	/	/
Co-loaded hybrid SLN-liposomes	350 ± 9	0.45 ± 0.05	-40.8 ± 5.1	91.4 ± 0.2*	2.7 ± 0.1$^{\#}$	100 ± 0.2*	1.9 ± 0.1§

*EE% was calculated considering the actual loading of SLNs used in the formulation of the hybrid systems. DL% was calculated knowing the weight of SLNs in 3 mL of SLNs suspension added. $^{\#}$ $p < 0.01$ between SLNs and liposomes and $p < 0.001$ within hybrid SLN-liposomes and the other nanocarriers; $^{@}$ $p < 0.01$ between SLNs and liposomes; § $p < 0.01$ between SLNs and liposomes and $p < 0.001$ within hybrid SLN-liposomes and the other nanocarriers.

3.2 AFM analysis

A detailed AFM analysis was carried out to examine the morphological difference between the formulations. The morphology of unloaded SLNs, liposomes, and hybrid SLN-liposomes was assessed by AFM. As shown by the topographic images (Fig. 1), all the examined particles exhibited a spherical shape, confirming that the preparation of the hybrid system did not significantly affect the liposomal morphology. Phase images provided complementary information, revealing variations in certain surface properties of the nanocarriers, such as viscosity, elasticity, and viscoelasticity. These deviations are recorded by the probe, which detects phase signal changes, resulting from regions of different composition. These phase shifts are visualized as bright and dark areas in the image, allowing the differentiation of materials. In detail, stiffer domains have a more positive phase shift, thus appearing as brighter than soft domains [19]. As can be noticed in the AMF images, the size of particles seems larger than that measured by PCS, especially for hybrid liposomes. This finding can

be explained considering that AFM images can be affected by the movement of the cantilever probe, which, by pushing and warming the particles, determines a deformation of their original morphology [20].

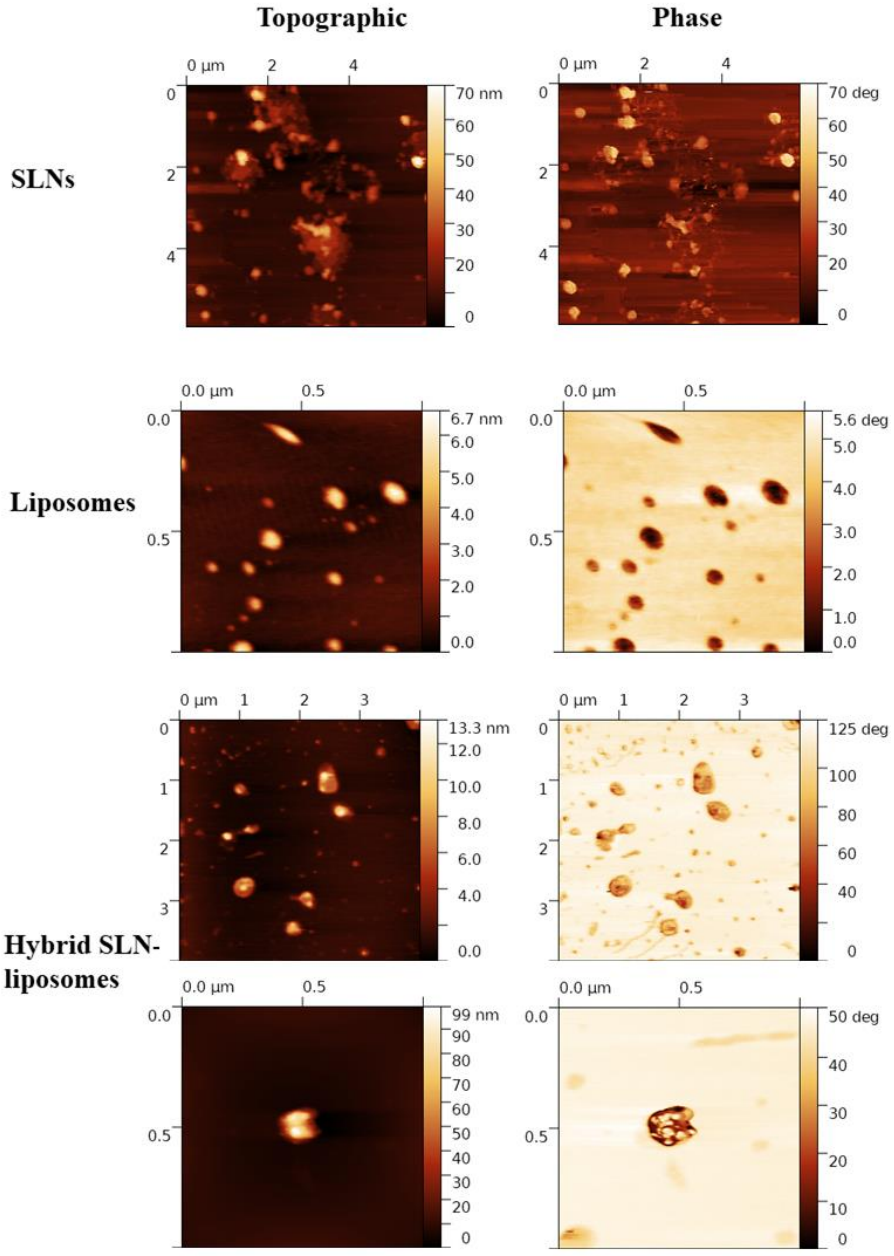

Figure 1. Representative AFM images of SLNs, liposomes, and hybrid SLN-liposomes. The topographic images of the samples are depicted on the left, while the phase-signal images are on the right.

3.3 SANS analysis

The SANS investigation was instrumental to the thorough characterization of the hybrid nanocarriers. In particular, it was useful to obtain further indications of the successful formation of the hybrid systems and to study the localization of the drugs in the particles.

Firstly, plain liposomes were investigated by SANS to obtain a fully characterized model for the comparative study of our hybrid systems (Figure 2).

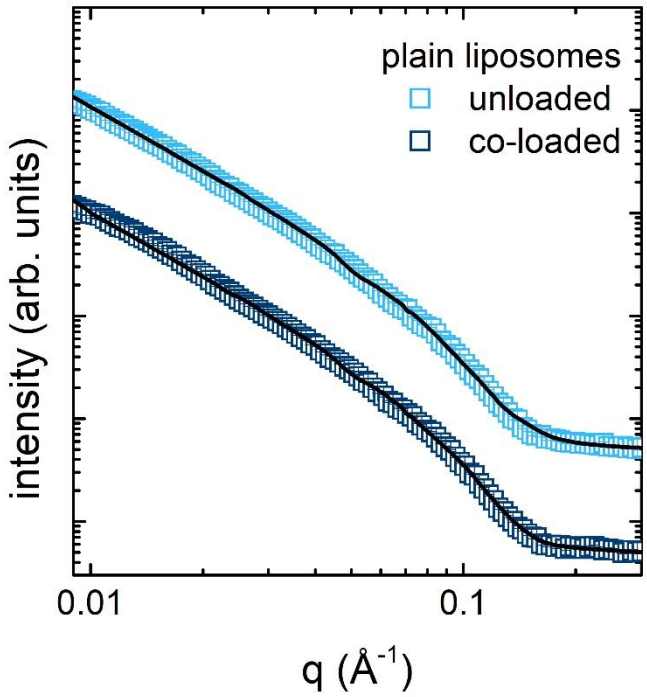

Figure 2. SANS scattering profile of plain liposomes: unloaded liposomes dispersed in D_2O (light blue squares) and co-loaded liposomes with both INH and RIF dispersed in D_2O (dark blue points). Best fits (solid lines) are reported in black. The curves are vertically shifted to better visualize the differences between the trends.

Liposome samples co-loaded with RIF and INH showed a slight increase in the lipid shell scattering length density (SLD, ρ_s) and a concomitant increment of the membrane thickness *t*, compared to unloaded liposomes (Table 2). This result was compatible with the encapsulation of the lipophilic RIF, which determines a rearrangement of the lipids in the single bilayer. A decrease in the SLD of the aqueous core, ascribable to the presence of INH, was also observed.

Table 2. Fit parameters for plain liposomes: ρ_c, SLD of the core; ρ_s, SLD of the shell, r_c, radius of the core; t, thickness of the shell.

Formulation	ρ_c [$\cdot 10^{-6}$ Å^{-2}]	ρ_s [$\cdot 10^{-6}$ Å^{-2}]	r_c (nm)*	t (nm)*	size (nm)
Unloaded liposomes	6.40	2.97	202 ± 1 [0.3]	3.3 ± 0.1 [0.3]	410 ± 2
Co-loaded liposomes	6.38	3.48	197 ± 1 [0.3]	3.5 ± 0.1 [0.2]	401 ± 2

* The corresponding polydispersity values assuming a Gaussian distribution are reported in square brackets.

Subsequently, we characterized our new hybrid systems, unloaded and co-loaded with the two selected drugs. The outcomes of the SANS analysis are shown in Figure 3, where they were compared with the data of the SLNs.

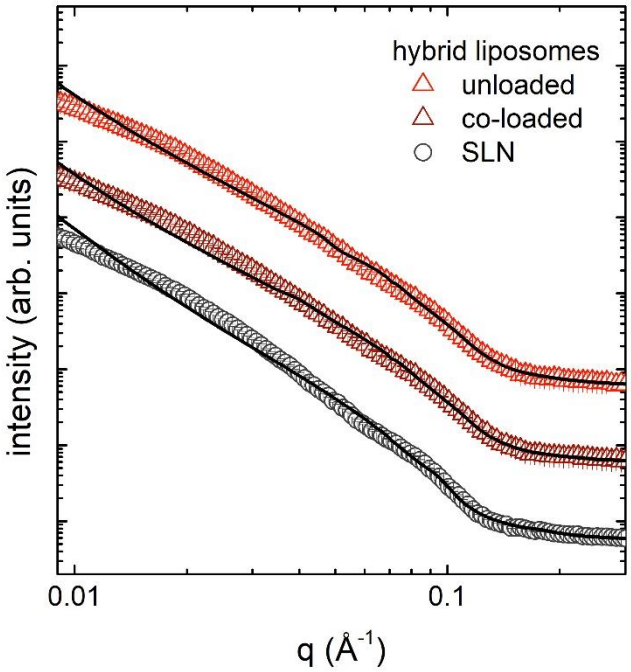

Figure 3. SANS scattering profile of SLNs and hybrid liposomes: SLNs (grey circles), unloaded hybrid liposomes (orange triangles) and co-loaded hybrid liposomes with both INH and RIF (red triangles). Best fits (solid lines) are reported in black. The curves are vertically shifted to better visualize the differences between the trends.

The dimensions of the unloaded hybrid SLN-liposomes (Table 3) were slightly bigger with respect to those of the corresponding unloaded liposomes (Table 2). This result may indicate the presence of the SLNs embedded in the core of the hybrid systems.

Table 3. Fit parameters for SLNs and hybrid liposomes: ρ_c, SLD of the core; ρ_s, SLD of the shell, r_c, radius of the core; t, thickness of the shell.

Formulation	ρ_c [$\cdot 10^{-6}$ Å^{-2}]	ρ_s [$\cdot 10^{-6}$ Å^{-2}]	r_c (nm)*	t (nm)*	size (nm)
Unloaded SLNs	0.59	-2.17	66 ± 1 [0.3]	4.3 ± 0.5 [0.3]	140 ± 4
Unloaded hybrid SLN-liposomes	5.11	2.95	203 ± 2 [0.2]	3.9 ± 0.2 [0.3]	414 ± 6
Co-loaded hybrid SLN-liposomes	5.15	3.01	206 ± 2 [0.2]	3.8 ± 0.1 [0.3]	419 ± 6

* The corresponding polydispersity values assuming a Gaussian distribution are reported in square brackets.

The analysis of the co-loaded hybrid SLN-liposome formulation also revealed a decrease in the SLD of the lipid shell (ρ_s) in the presence of INH and RIF (compared to the samples without SLN in Table 2). A comparative significant decrease in the SLD of the core (ρ_c) was observed for both unloaded and co-loaded hybrid SLN-liposomes, further indicating the encapsulation of SLNs in the aqueous core. More details about the shell and core SLD trends and their correlation with the drugs localization are reported in the discussion section.

Notably, although the size of unloaded hybrid SLN-liposomes was comparable to that of unloaded plain liposomes, the dimensions of the co-loaded systems differ from each other by approximately 20 nm.

3.4 In vitro release study

To determine if these formulations exhibited different release kinetics, an *in vitro* release study was carried out, comparing the results to those obtained for the diffusion of the free drugs through a dialysis bag. All experiments were performed in triplicate in a time frame of 1-180 min, at 37 °C. The resulting kinetic profiles are depicted in Figure 4.

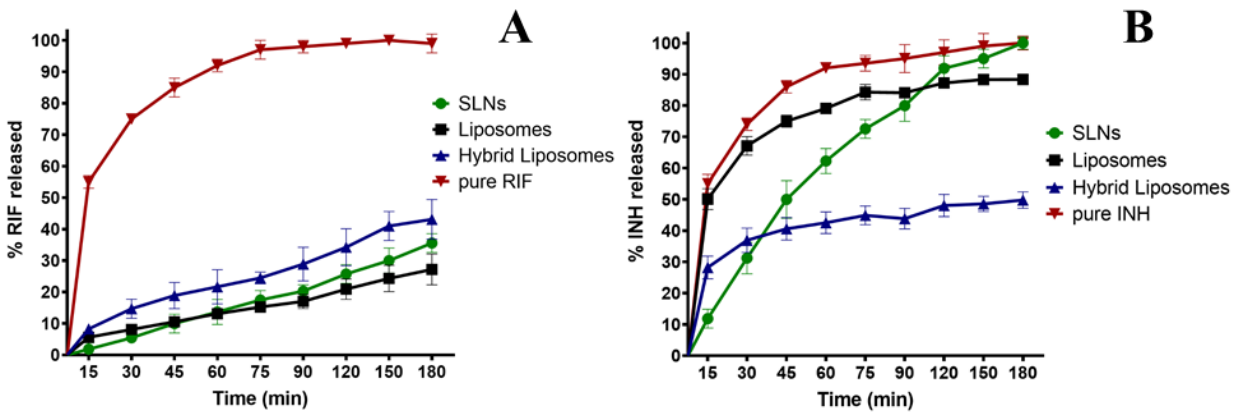

Figure 4. *In vitro* release of RIF (A) and INH (B) from co-loaded SLNs, liposomes, and hybrid SLN-liposomes.

Concerning the release curve of RIF, almost 25% of the drug was released from plain liposomes after 180 min. This value increased to 45% for the hybrid carriers. After a slight initial burst, like that of liposomes, the hybrid systems exhibited a release rate comparable to that of SLNs. This observation may indicate that the presence of the SLNs dictates the overall rate of the system; conversely, a slightly but significantly different profile was detected for plain liposomes.

Also for the hydrophilic IHN, huge differences in the release rate were observed: our data showed that SLNs released 100% of the drug in 3 h. On the contrary, the release from liposomes was slower: after a sharp burst effect, corresponding to 50% of INH released in 15 min, a plateau at 80% was reached, which lasted for the remainder of the experiment. Finally, hybrid SLN-liposomes released only 50% of the drug in 3 h, demonstrating an optimal ability to control the drug release.

4. DISCUSSION

Due to their great advantages, SLNs are attracting the attention of researchers worldwide for their potential application in the pulmonary delivery of antitubercular drugs. However, one of the main drawbacks of SLNs is their inability to stably incorporate hydrophilic drugs [4,21]. In our previous work [9], liposomes proved to be suitable systems for the delivery of both hydrophilic and lipophilic drugs, such as INH and RIF, respectively.

In the present work, the development of a hybrid SLN-liposome system was attempted to design a novel strategy for a potential successful pulmonary delivery and controlled release of INH and RIF. The newly developed hybrid nanocarrier was compared to plain co-loaded SLNs and liposomes, in terms of size, morphology, structure, and *in vitro* release of both drugs.

Liposomes and hybrid SLN-liposome systems were formulated by using lecithin as mixture of phospholipids to construct a bilayer shell, differently to our previous work, in which phosphatidylcholine (PC) was employed. Lecithin was preferred over PC in view of obtaining a therapeutic advantage for the management of TB. In addition to its surfactant properties, which are highly desirable for pulmonary medications, lecithin confers a negative charge to the liposomes, enhancing and sustaining the release of RIF in macrophages. [22],[23].

The characterization of the hybrid nanocarriers was initially performed by PCS, and the outcomes were compared to images obtained by AFM. From the PCS analysis, liposomes and hybrid SLN-liposomes displayed comparable particle sizes (~ 300 nm), while SLNs showed a smaller particle size (~130 nm), approximately half of that of liposomes. Despite the similar dimension, plain liposomes and hybrid systems differed for the particle homogeneity, as suggested by the increment of the PDI value, which increased from 0.3 to 0.45 in the case of the hybrid systems. The AFM images (Figure 1) confirmed that the incorporation of the SLNs into the liposomes did not result in a significant size change with respect to plain liposomes. In AFM images, liposomes appeared as dark spots in the phase-images, due to their soft consistency; on the contrary, hybrid SLN-liposomes appear as light-colored structures, owing to the presence of smaller SLNs in the core with a stiffer domain. Moreover, the presence of small bright spots due to non-incorporated SLNs, may explain the high PDI value recorded by PCS.

Concerning the drug loading investigation (Table 1), a high EE% was recorded for RIF in all the formulations, due to its hydrophobic interaction with the lipid matrix of SLNs and/or the lecithin bilayers of both liposomes and hybrid SLN-liposomes. As expected, RIF content in plain lecithin liposomes was lower than that of the PC-based liposomes of our previous work [9], despite they were prepared with the same formulation parameters. This difference can be attributable to the chemical nature of lecithin, which is composed by several phospholipids, including negatively charged phospholipids (e.g. phosphatidylinositol). It is known that in liposomes composed by phospholipids with negatively charged head groups, RIF localizes in a more superficial region, near the membrane interface, because of electrostatic repulsions between the anionic lipid and the ionized form of RIF [15]. Rodrigues *et al.* noticed that RIF can interact with various areas of the lipid bilayer with different partition coefficients, depending on the electrostatic/hydrophobic characteristics of the liposomes. In our case, plain lecithin liposomes showed a more negative Z-potential (-40.8 ± 5.4 mV) with respect to PC-liposomes (-20.5 ± 5.7 [24]), determining a repulsive electrostatic interference with RIF and concurring to its low incorporation. Moreover, the more negative charge of lecithin liposomes compared to PC liposomes also induced structural differences. For instance, the SANS scattering profile of the lecithin-based carriers did not exhibit the distinctive Bragg diffraction peak typical of

multilamellar assemblies, suggesting a unilamellar structure, probably due to repulsive interaction between negative phospholipids [25]. Moreover, the size of liposomes co-loaded with INH and RIF differed from that of the hybrid formulations by about 20 nm. As previously observed in the case of multilamellar liposomes, the co-loading determines a stabilizing effect even in the case of unilamellar liposomes, coherent with the decrease in the liposome radius observed for liposomes in absence of SLN [9]. The presence of SLNs in the new hybrid systems might exert an analogous stabilizing effect, resulting in an overall similar behaviour.

Regarding the hybrid SLN-liposomes, the drug EE% was calculated by considering the drug loading in the SLNs employed in the formulation. Therefore, the EE of RIF (91.4%) suggested that part of the hydrophobic drug was lost during the preparation of the hybrid nanocarriers through the reverse-phase evaporation method. This minimal loss of RIF might be due to the organic solvent (ether) used to obtain the water-in-oil emulsion during the formulation process, which could extract RIF from the SLN lipid matrix. Besides providing invaluable information on the size and thickness of the drug carriers, the SANS analysis allowed to ascertain the presence of the drugs in the lipid structure. In accordance with the hypothesis formulated after the encapsulation analysis, evidence of the entrapment of RIF in the shell of the hybrid SLN-liposomes was revealed by an increase in the SLD of the lipid shell (ρ_s=3.01) upon drug loading with respect to unloaded liposomes (ρ_s =2.95). This effect is due to the inclusion of RIF molecules which, even in low quantities, induce a less dense, less compact, and more disordered packing of the lipid shell [9]. This might alter the stability of the lipid shell and even favor the release of RIF molecules in simulated lung fluid (SLF).

As far as the hydrophilic INH is concerned, SLNs demonstrated a lower EE% with respect to plain liposomes, due to the low dispersibility of the drug in the lipid matrix. The incorporation of SLNs in liposomes did not induce any loss of INH during the formulation process, as confirmed by the fact that hybrid SLN-liposomes displayed an EE% value near 100%. This evidence highlighted the superiority of our hybrid systems in obtaining a high incorporation efficiency, considering that both the drugs were successfully incorporated into the final hybrid nanosystem. This feature has been observed in other hybrid systems recently reported in the literature [26,27].

The release of RIF from all the nanocarriers was slower than the diffusion of the free drug through the dialysis bag (Figure 4A), highlighting a sustained release pattern from all the lipid-based nanocarriers. The slower release of RIF may be attributable to the interaction with either the solid lipid matrix of SLNs or the hydrophobic tails of the liposomal bilayer, suggesting that in any case RIF was stably incorporated into the carriers [9,28]. *In vitro* sustained-release of RIF from hybrid SLN-liposomes was slightly higher than that observed for plain liposomes and SLNs. This difference could be due to the ether-induced extraction of RIF from the SLN matrix during the formulation

process, as discussed for the encapsulation studies. The portion of RIF extracted during the water-in-oil emulsion preparation could be absorbed in the outer layer of the phospholipid shell. As shown by the release profiles depicted in Figure 4, the highest percentage release of RIF was observed for the hybrid systems (which retained more of it in terms of EE%, both in the core and in the shell), followed by SLNs (only in the core), and liposomes (only in the shell).

Diffusion of free INH across the dialysis membrane indicated that the entire drug was dissolved in < 60 min (Figure 4B). SLNs slowly released the drug with quite a linear profile, reaching 100% in 4 h. On the contrary, liposomes, being a vesicular system, released the hydrophilic drug with a profile similar to that of the free drug, probably due to the high permeability of the unilamellar liposomal membrane [29]. Hybrid SLN-liposomes showed a typical biphasic release profile, with a burst release in the first 30 min followed by a sustained release in the next 3 h. The decrease in the SLD of the core of hybrid liposomes witnesses the incorporation of INH and SLNs, with a high EE%. The two compounds indeed show SLDs that are lower compared to that of D2O, due to their molecular composition. Hence, INH was trapped in the SLNs embedded in the core of the hybrid systems, which enhanced its retention in SLF with respect to plain liposomes, resulting in a unique release profile. In agreement with previous data [30], this finding suggested that hybrid systems have the potential to delay the release of inhaled drugs from different matrices due to the presence of a diffusional barrier on the nanoparticle surface.

CONCLUSIONS

The present investigation highlights the prospects of nanoscale shell-core SLN-liposome hybrids as efficient carriers for a potential pulmonary delivery of antitubercular drugs. This formulation prevents the leakage of small hydrophilic compounds and increases the entrapment of drugs. These novel systems were able to release their content as expected from a successful dosage form for inhaled administration, improving the stability and the drug release profile with respect to plain liposomes. The physicochemical characterization of our systems opens new avenues towards a better understanding of the formulation of vesicles encapsulating SLNs. The SANS analysis allowed us to precisely define the variation in size of the nanosystems after the encapsulation of co-loaded SLNs The *in vitro* dissolution studies depicted an initial burst release followed by a sustained release profile, significantly slower compared to that of plain liposomes.

We can conclude that the hybrid system designed and characterized in this work offers various benefits with respect to conventional nanocarriers, such as a high encapsulation efficiency, a controlled particle size, and the potential to load multiple therapeutic agents, determining a prolonged release of the drug. Respirability parameters as well as biological studies *in vitro* and *in vivo* will be

planned to confirm the advantages of this hybrid SLN-liposomal system in the treatment of lung infections.

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Declaration of interests

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Design and physicochemical characterization of novel hybrid SLN-liposome nanocarriers for the smart co-delivery of two antitubercular drugs

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☒ 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.

Author statement file

E. Leo, F. Domenici, and C Castellano conceptualization and supervision; E. Truzzi, A. Capocefalo and C. Castellano: investigation and formal analysis; E. Truzzi, M. Mori and E. Maretti: metodology and data curation. A. Capocefalo and F. Domenici: Softwere; E. Leo and F. Meneghetti writing original draft; F. Domenici, and V.Iannuccelli: Writing - review & editing. E.Leo, F. Meneghetti, C.Castellano and F. Domenici: funding acquisition.

All authors have read and agreed to the published version of the manuscript.