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Research paper

Drug delivery of rifampicin by natural micelles based on inulin: Physicochemical properties, antibacterial activity and human macrophages uptake

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ABSTRACT

This work aims at designing a drug delivery system for rifampicin (RIF) to be used for the therapy of infections from *mycobacterium tuberculosis* or other lung-colonizing bacteria. We are proposing, in particular, the delivery of RIF by micelles based on inulin functionalized with vitamin E (INVITE). We previously demonstrated that INVITE micelles are formed from the self-assembling sustained by the interaction, within the hydrophobic core, of aromatic groups belonging to vitamin E. It points on the effectiveness of these biocompatible systems in incorporating aromatic-group-bearing hydrophobic drug such as RIF. The succinilated derivative of INVITE, namely INVITESA, was further studied. Other than a full physicochemical characterization, the obtained micelles containing RIF were tested for their antibacterial activity against Gram- or Gram + bacteria including *mycobacterium* smegmatis. Furthermore, uptake studies on human alveolar macrophages and MTT studies were performed.

1. Introduction

Mycobacterium tuberculosis has colonized humans since the beginning of their history. It causes the tuberculosis (TB) that, even today, is hard to treat although effective antibiotics are available. The difficulties on the treatment arise from drug resistance phenomena and from a natural protection of mycobacteria against external substances. It has been calculated that in 2012 TB caused 1.3 million of deaths worldwide [1]. One of the main cause for the incurrence of drug resistance is a low adherence of the patient to the treatment that should be carried on for approximately nine months. It has been reported that: "The repeated use of the same drugs, together with prolonged regimens that often lead to poor patient compliance, has resulted in the emergence of strains that are increasingly resistant to the available drugs." [2]. Rifampicin (RIF) is one the most effective drugs used for TB therapy but, as mentioned before, its administration, in combination with other drugs, must be repeated for a long time, even months. The way *mycobacterium* colonizes the host is a major factor regarding TB spreading and the difficulties in treating the infection. In fact, mycobacterium survives within human macrophages, escaping the normal mechanisms the macrophages use to eliminate "normal" bacteria. The mechanisms involved in this long staying and escaping are well illustrated in several scientific works [3].

From these intrinsic characteristics of *mycobacterium* arise the need of finding new drug delivery systems for antibacterial drugs such as RIF that could reach the site of infection and still maintain the antibacterial activity of the free drug. A well-designed drug delivery system (DDS) should promote the accumulation of the drug directly at the site of the infection providing a controlled release of the drug and, consequently, promoting the accumulation and activity of intracellular antibiotic drugs. It would allow a reduced number of administrations by still maintaining the therapeutic effectiveness of the drug and reducing the side effects [4].

By a pharmaceutical point of view, a problem in using RIF in therapy is its high hydrophobicity which requires the use of specifically designed drug delivery systems to be resolved. Among the possible approaches, the dispersion of RIF within nanoparticle or micelles is promising. Polymeric micelles, in particular, as self-assembling systems, could show several advantages with respect to classic nanoparticles made from, e.g., PLA or PLGA. Going deeper in this concept, we think that polysaccharides have a number of advantages upon polyesters such as the presence of different sites of derivatization and, thus, the

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possibility to form amphiphilic species by simple functionalization with hydrophobic moieties, though, still exposing a hydrophilic shell to the aqueous environment. To corroborate this approach, different studies on polymeric micelles incorporating RIF have been published lately. For example, Gao et al., prepared polymeric micelles from hyaluronic acid functionalized with tocopherol succinate drug loaded with RIF. They obtained micelles with a hydrodynamic size at around 250 nm in which drug release was accomplished within hours. The study was further completed with in-vitro studies on murine alveolar macrophage [5]. Moretton et al., prepared polymeric micelles from a co-polymer made from poly(ε-caprolactone)-b-poly(ethylene glycol)-poly(ε-caprolactone). In particular, they studied their systems by a pharmaceutical point of view by assessing the main formulations to obtain a stable and re-dispersible formulation of RIF [6,7]. Here we are proposing the micelle system formed from inulin and vitamin E (INVITE) and its succinilated derivative (INVITESA) as drug delivery system for RIF. In the last years, the chemical functionalization of INU with different hydrophobic moieties, led to amphiphilic derivatives with favorable properties. For example, Morros at co-workers, prepared octenyl and dodecenyl succinic esters of inulin with the aim to form INU based surfactant as not-petroleum derived molecules [8]. In the same way, Kurecic and co-workers, used a commercially available surfactant based on INU for the stabilization of clay minerals [9]. However, a great part of INU based amphiphiles in literature, are used in the pharmaceutical field, in particular, in the formation of polymeric micelles as DDS [10–12].

Based on our previous findings, INVITE systems are able to load hydrophobic drug; with particular emphasis on aromatic-group-bearing molecules [13]. We also found that INVITE micelles are promptly internalized by different cell lines within a few minutes and were highly biocompatible toward the same cell lines. Furthermore, inulin, as the constitutive backbone exposed to the aqueous environment, does not bind to plasmatic proteins, which is considered as one of the causes that can prevent the nanosystems from their cellular uptake by macrophages [14].

2. Materials and Methods

2.1. Chemicals

All reagents were of analytical grade, unless otherwise stated.

N,N-dimethylformamide (DMF), succinic anhydride (SA), triethylamine (TEA), diciclohexylcarbodiimide (DCC), D-α-tocopherol succinate, NaCl, KCl, Na_2HPO_4, KH_2PO_4, D_2O, DMSO-d_6, diethyl ether, fluorescein isothiocyanate (FITC), mucin from porcine stomach type II, polyacrylic acid (Mw 5000 Da) and rifampicin (RIF) were purchased from Sigma-Aldrich (Milano, Italy). Inulin from dahlia tubers (INU, approx. 5000 Da), N-Hydroxysulfosuccinimide sodium salt (NHSS), Polisorbate 80 were purchased from Fluka (Milano, Italy). DMSO was purchased from Carlo Erba Reagents (Milano, Italy). Dialysis tubes with a MWCO 3.500 Da (Spectra/Por® 6) were purchased from Spectrum Labs.

2.2. Apparatus

FT-IR spectra were recorded in the range 4000–400 cm^{-1} using a Perkin-Elmer 1600 IR Fourier Transform Spectrophotometer (Monza, Italy). The resolution was 4 cm^{-1}.

UV–vis analyses were performed by using a Perkin–Elmer Spectrometer Lambda 25, Perkin-Elmer, (Monza, Italy).

^{1}H NMR spectra were recorded using a Varian Mercury 300 MHz instrument.

Centrifugations were performed with a Beckman Avanti 30 (Milano, Italy) equipped with a temperature control.

Lyophilizations were performed by a Christ Alpha 1–4 LSC instrument at −59 °C and 0.016 mbar.

The mean size, polydispersity index (PDI) and zeta potential of the unloaded or rifampicin loaded micelles were measured by using a ZetasizerNano ZS (Malvern Instruments Ltd., Worcestershire, UK).

2.3. Synthesis and physicochemical characterizations

2.3.1. Synthesis of inulin (INU)-vitamin E (VITE) bioconjugate, INVITE

The INU-VITE bioconjugate (INVITE) was synthesized as previously reported [15].

Briefly, carboxyl group of VITE succinate (1 eq) was activated in anhydrous DMF under nitrogen with DCC (2 eq) and NHSS (2 eq) under stirring for 3 h at 25 °C. To this mixture, INU was added together with TEA as DMF solution with the following molar ratio: moles of VITE/moles of INU RU = 0.2; moles of TEA/moles of INU RU = 0.1, the reaction was carried out under nitrogen at 25 °C for 12 h. After this time, the reaction mixture was precipitated and purified in diethyl ether. The resulting powder was dried under reduced pressure at 25 °C. Derivatization degree (DD) was determined by ^{1}H NMR (DMSO-d_6) as rate between the integral of the peak centered at ≈ 0.87 ppm (m, 12H, 4 × CH_3 alkyl chain VITE) and the integrals of the peaks comprised between ppm 3.49–4.05 belonging to the seven protons of INU fructose ring. DD 19.5% (mol/mol).

FT-IR and ^{1}H NMR data were in agreement with previous results [13,15].

2.3.2. Synthesis of INVITE-succinate bioconjugate, INVITESA

INVITESA conjugate was synthesized by following a reported method [16,17].

INVITE bioconjugate was solubilized in anhydrous DMF, then, trimethylamine (TEA) and succinic anhydride (SA) were added: moles of SA/moles of INVITE RU = 0.4; moles of TEA/moles of INVITE RU = 0.5. The reaction was carried out under stirring and nitrogen for 20 h at 25 °C. The product was recovered and washed in diethyl ether. Finally, the gained dry powder after vacuum drying was subjected to dialysis (3500 Da MWCO, Spectra/Por® 6) and lyophilized.

DD was calculated by ^{1}H NMR in D_2O since this solvent allows the resolution of succinic peaks [13].

Succinic protons were found at ppm 2.56 (s, 2H, CH_2 succinic chain inulin, carboxyl side), 2.39 (s, 2H, CH_2 succinic chain inulin, ester side) DD % in SA resulted 38% (mol/mol). FT-IR and ^{1}H NMR data were in agreement with previous results [16].

2.3.3. Synthesis of INVITE or INVITESA conjugate with FITC

INVITE or INVITESA conjugated with FITC were prepared as previously reported, with some modification [16]. Briefly, INVITE or INVITESA were solubilized in anhydrous DMF and FITC was added with a molar ratio moles of FITC/moles of INU RU = 0.01. The flask was protected from light with aluminum foil. After 3 h, the reaction mixture was recovered and washed in ice-cold diethyl ether. The powder obtained after under-vacuum drying was solubilized in DMSO, dialyzed against double distilled (MWCO 3.500 Da, Spectra/Por® 6) for 2 days in the dark and, finally, lyophilized. ^{1}H NMR in DMSO-d_6 was in agreement with the literature [16,18].

The DD % in FITC was ≈ 1% (mol/mol).

2.3.4. Formation of empty INVITE or INVITESA micelles and rifampicin (RIF) loaded INVITE or INVITESA micelles

Empty micelles from INVITE or INVITESA or loaded with rifampicin and called INVITE-RIF or INVITESA-RIF were prepared by the dialysis method. To assure the RIF loading within the micelles, the polymers concentration was always maintained above their critical association concentration (CAC) ($7.5 \cdot 10^{-3}$ mM for INVITE and $9.1 \cdot 10^{-3}$ mM for INVITESA).

Calculated amounts of INVITE or INVITESA were dissolved in dimethylsulfoxide with or without RIF (10% w/w) with respect to the polymer and left under constant stirring for 1 h. The polymeric dispersions were poured into a dialysis tubes (MWCO 3.500 Da, Spectra/

Por® 6) and dialysis was carried out in the dark for 3 days against distilled water. The final solution was freeze-dried giving a white or red powder for unloaded or RIF loaded micelles, respectively.

2.3.5. Evaluation of drug loading and encapsulation efficiency of RIF loaded micelles

For the evaluation of drug loading, 2 mg of lyophilized INVITE-RIF or INVITESA-RIF were dissolved in 1 mL of DMSO and left under constant stirring for 1 h. Then, the solution was 10-fold diluted with the same solvent and the amount of loaded RIF was determined by a UV–Vis spectrophotometry by reading at 480 nm. Each measurement has been performed in triplicate. The loaded amount of RIF was calculated by a calibration curve of RIF in DMSO in the concentration range 5–60 μg/mL (correlation coefficient r2 > 0.999).

The RIF loading (DL w/w %) and the encapsulation efficiency (EE w/w %) on micelles were calculated according to the Eqs. (1) and (2):

$$DL\%(p/p) = (\text{amount of RIF in INVITE micelles/starting weight RIF} + \text{micelles}) \times 100 \quad (1)$$

$$EE\%(p/p) = (\text{Experimental DL/Theoretical DL}) \times 100 \quad (2)$$

2.3.6. Size distribution, zeta potential and size stability over the time of empty or RIF loaded micelles

A dynamic light scattering study was performed.

In particular, aqueous solutions of INVITE, INVITESA, INVITE-RIF OR INVITESA-RIF, 1 mg/ml, were prepared and left to equilibrate overnight at 25 °C with gentle stirring. Then, the micellar dispersions were filtered on a 0.45 μm filter and analyzed by ZetasizerNano ZS instrument. The measurements were performed in triplicate for each sample.

Moreover, unloaded and RIF-loaded INVITE or INVITESA aqueous solutions were used to define the Z-potential of the micelles at 1 mg/ml added of KCl 1 mM.

Unloaded and RIF-loaded INVITE or INVITESA 1 mg/ml dispersions were prepared in water/NaCl 0.9% w/v at 25 °C to test the size stability of the micelles up to 60 days. The measurements were carried out in triplicate and reported as mean ± SD.

2.3.7. Mucin-INVITE/INVITESA/INVITE-RIF/INVITESA-Rif interaction studies by transmittance measurement (turbidimetric study); mucoadhesion

Mucin dispersion was prepared by dissolving it in PBS pH 6.8, final concentration 0.1% w/v. The suspension was allowed to equilibrate for 24 h at 37 °C. INVITE, INVITESA, INVITE-RIF or INVITESA were added to this mixture, 10 mg of each sample in 10 mL of mucin dispersion were used. Polyacrylic acid was used as positive control (mucoadhesive), its Mw was 5000 Da, similar to INU. At established time points 2, 4, 6, 24 h samples' transmittance was evaluated at 650 nm.

All measurements were performed in triplicate and expressed as mean ± SD.

2.3.8. Drug release studies from INVITE-RIF or INVITESA-RIF

In vitro release studies were performed by the dialysis method [19,20]. In particular, 20 mg of INVITE-RIF or INVITESE-RIF were solubilized in 1 mL of double distilled water and the solutions poured into a 3500 Da MWCO dialysis membrane. The dialysis tube was incubated in 20 mL of PBS pH 7.4 containing polysorbate 80 (0.5% v/v) at 37 °C under stirring (100 rpm).

At established release points, the entire release medium was removed and replaced by pre-warmed fresh release media. The amount of released RIF has been determined by UV–Vis spectrophotometry at 480 nm by comparing the data with a calibration curve of RIF performed in the same medium in the concentration range 5–60 μg/mL (correlation coefficient r2 > 0.999. All analyses were carried out in triplicate and all data were expressed as mean ± SD.

2.4. Antibacterial activity

2.4.1. Evaluation of the antibacterial activity of empty or drug loaded micelles on lung-colonizing selected bacteria

The following strains were used for testing the antimicrobial activity of empty or RIF loaded micelles: *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 10145 and *Mycobacterium smegmatis* ATCC 14468. Bacteria were cultured in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) at 37 °C, in Tryptone Soya Broth with defibrinated sheep blood for M. smegmatis at 37 °C and in Todd Hewitt Broth (THB,Oxoid, Basingstoke, UK) at 37 °C in the presence of 5% CO_2 for S. pyogenes. The bacteria cultures were centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in phosphate buffered saline (PBS, pH 7.3). The suspension was diluted to adjust the number of cells to $1{\cdot}10^7$–$1{\cdot}10^8$ colony forming units (CFU)/ml.

2.4.2. Evaluation of the minimum inhibitory concentration (MIC)

Dispersions of empty or rifampicin loaded micelles were obtained in distilled water. The antimicrobial activity of INVITE and INVITESA systems was determined with the macrodilution broth method, according to: Clinical and Laboratory Standards Institute, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically-Eighth Edition: Approved Standard M7-A8, Wayne, PA, USA (Clinical and Laboratory Standards Institute, 2009), with some modifications reported in this paragraph. The desired concentration was obtained by adding the appropriate volume of the drug to a given volume of Iso-Sensitest broth (ISB, Oxoid) in 15 mm × 100 mm tubes, inoculated with bacterial suspensions with a concentration of 107 CFU/ml. The minimum inhibitory concentration (MIC) was evaluated after a 24–48 h incubation at 37 °C, as the lowest concentration that completely inhibited the formation of visible microbial growth. Stock standard solution of rifampicin was used as positive control. Control test tubes containing broth without drug solution for each organism tested were used (negative control).

All the experiments were performed in duplicate and bacteria-free broth was included as culture control. The activity of empty micelles (without rifampicin) was also tested.

2.5. In-vitro studies on human alveolar macrophages

2.5.1. Isolation and culture of human alveolar macrophages

Human alveolar macrophages were isolated from bronchoalveolar lavage (BAL); lavage fluid was centrifuged and BAL cells were seeded into 96-well plates (10000 cell/well) using RPMI 1640 medium +10% FBS 1% L-glutamine +1% Pen/Strep +1% Amphotericin B. After 24 h, each well was washed with PBS solution to remove non-adherent cells and to select only alveolar macrophages. BAL donors gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.

2.5.2. Micelles uptake by alveolar Macrophages

Macrophages were incubated with INVITE-FITC and INVITESA-FITC micelles (0.5, 1 and 2 mg/ml) for 30 and 120 min. After the incubation time, cells were washed with PBS solution and the cellular uptake was evaluated using a microplate reader (Synergy HT, BioTek, United Kingdom), equipped by an excitation filter at 485 nm and an emission filter at 528 nm, exploiting the FITC fluorescence property. Untreated cells were considered as negative control. Each condition was performed in triplicate on two cell lines (from two different donors).

2.5.3. Cell viability evaluation by MTT test

Macrophages were exposed to INVITE-FITC and INVITESA-FITC micelles (0.5, 1 and 2 mg/ml) for 24 h. After incubation time, samples

were removed and 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) were added in each well. MTT solution was removed after three hours and 100 µl of DMSO were added. Optical density was measured on Synergy HT at 570 nm and 670 nm (reference wavelength). Each condition was tested in triplicate. Cell viability (%) was calculated: 100 × (ODs/ODc), where ODs is the mean value of the measured optical density of the tested sample and ODc is the mean value of the measured optical density of untreated cells (control).

3. Results and discussion

Recently, we developed a drug delivery platform based on inulin functionalized with vitamin E. Since inulin represents the hydrophilic part of the bioconjugate while vitamin E is the hydrophobic part, the resulting product is an amphiphile which forms polymeric micelles upon water dispersion. Among the main features of these products, called INVITE, we found they show a particular spatial disposition upon self-assembling. In particular, it has been found that the alkyl chain of vitamin E residues are exposed to the outer side of the micelle interpenetrating the inulin layer while the hydrophobic core is formed by π-π stacking of aromatic groups in vitamin E [13]. This finding let us to hypothesize that the external surface of INVITE micelles, even if mostly hydrophilic, could show a certain affinity to hydrophobic or partially-hydrophobic surfaces or substances such as bacterial/cellular surfaces. Furthermore, the mentioned core formation by π- π stacking allows an effective drug loading of aromatic group-bearing molecules [16,21]. INVITE systems were also useful in the co-delivery of hydrophilic and hydrophobic drugs by including them into hydrogels [22,23]. On the other side, we demonstrated that the cellular uptake of INVITE micelles is fast and complete in three different cell lines, namely, Caco-2, mesenchymal stromal cells and human embryonic kidney (HEK 293) cells [18,20,21]. Another important feature of INVITE micelles arises from their long-circulating behaviors (up to 72 h) as demonstrated *in-viv* [18].

Here, for the first time we are assessing the use of the INVITE platform for the drug delivery of one of the most important antibiotic used in the treatment of tuberculosis (TB), the rifampicin (RIF). While the delivery of this drug into INVITE micelles is predictable, what has to be addressed is the maintenance of the antibiotic activity which depends on micelle-bacterium interaction and on the release of the drug from the DDS. Furthermore, in view of a pulmonary administration of the DDS for the local treatment of TB, also the succinic derivative of INVITE (containing free carboxyl groups) was included in this study, mostly to exploit the possible more pronounced mucoadhesion properties of the carboxylated derivative that would increase the time of the residence of the DDS along the respiratory tract.

3.1. Physicochemical characterization of the studied amphiphilic biopolymers

3.1.1. ^{1}H NMR, FTIR, DLS (hydrodynamic size and size stability studies) and zeta potential

In this study, four different bioconjugates have been synthesized: INVITE, INVITESA, INVITE-FITC and INVITESA-FITC. These products were characterized by ^{1}H NMR, FTIR, DLS and zeta potential.

^{1}H NMR and FTIR studies were in agreement with our previous results [15,16]. In particular, ^{1}H NMR studies in DMSO-d_6 for INVITE showed, with respect to native INU, the new peaks at ppm ≈ 0.89 relative to the 12 protons of VITE alkyl chain and the peaks between ppm 2.01–1.02 relative to VITE aromatic groups and CH_3 residues of VITE alky chain. ^{1}H NMR spectrum of INVITESA was recorded in D_2O to discriminate between the succinic protons of succinic anhydride and those from succinic spacer of VITE. In fact, in a previous work we demonstrated that when the ^{1}H NMR of INVITE is recorded in D_2O, the succinic protons of VITE succinate being part of the hydrophobic core, cannot be visualized by ^{1}H NMR [13]. On the contrary, succinic protons from INVITESA, being on the outer hydrophilic layer, can be successfully detected and quantified. For this reason, the two peaks at ppm 2.54 and 2.43 were used to calculate the derivatization degree (DD) in SA that resulted 38 ± 1.3% mol/mol. For FITC derivatives of INVITE and INVITESA, the peaks at ppm 7.98 (1H, d, FITC), 7.65 (1H, m, FITC), 7.06–7.12 (3H, m, FITC), 6.85 (4H, m, FITC) were found as significant for assessing the chemical bond formation between the derivatives and FITC. In particular, the peak at ppm 7.98 was used to calculate the DD in FITC that resulted ≈ 0.9 ± 0.2% mol/mol for both INVITE and INVITESA. ^{1}H NMR was further used to demonstrate that after RIF loading, the drug can be found inside the micelles, which we successfully used this method for curcumin in a previous study [13]. The starting assumption is: when drug loaded micelles are solubilized in a good solvent for both polymer and drug, the system will be "molecularly" dispersed and all the protons will became detectable upon ^{1}H NMR analysis. On the contrary, when a solvent such as water determines the amphiphilic polymer self-assembling, the response to magnetic resonance inside the internal core will be so low that no NMR peaks will be detected. When this behavior is seen and the characteristic peaks of the drug are not detected, it will demonstrate that the drug is incorporated inside the hydrophobic core and not, e.g., adsorbed on the outer surface of the nanosystem, Fig. 1.

FTIR studies confirmed the presence of the carboxyl group in INVITESA due the appearance of the bands at 1572 and 1413 cm^{-1} attributable to, respectively, the ν_s and ν_{as} of carboxyl moieties.

Dynamic light scattering and zeta potential studies were performed to assess the main hydrodynamic size and surface charge of INVITE based nanomicelles. In Table 1 the mean hydrodynamic size, polydispersity index and zeta potential are shown.

The hydrodynamic size was expected to be lower for INVITESA samples as the more hydrophilic shell of these micelles, due to the exposure of carboxyl groups, determines a stronger packing of the hydrophobic core, though no significant differences could be evidenced in the size before and after drug loading. The slight differences are attributable to the experimental error and within the standard deviation. Zeta potential was significantly lower for INVITESA with respect to INVITE micelles. In particular, it resulted ≈ 60% lower indicating contributions of dissociated carboxyl groups on the zeta potential. This effect was strongly reduced by the drug loading with RIF. It could be attributed to the presence of the piperazine group belonging to RIF, with a pKa 7.9 [6,24]. As the piperazine group could be considered hydrophilic, it could be supposed that this group will be partially exposed, or close, to the surface of the micelle (also considering that it is far from the aromatic portion of RIF that establishes the aromatic interaction within the INVITE core). Since in water it will be protonated by the protons belonging to the micro-acidic environment of INVITESA, its main charge will be positive, thus, counterbalancing the negative charge from INVITESA carboxyl group. In fact, in INVITE micelles the zeta potential does not change upon drug loading because at neutral pH the piperazine group is not strong enough to hydrolize water, so no positive charges will contribute to change the negative zeta potential of the micelles.

DLS stability studies, Fig. 2, show an overall stability of the micelles up to 30 days since the micelles size resulted stable within the found values ± SD for the tested time points. Some increase in size could be evidenced after 45 days and, in particular, after 60 days especially for INVITESA-RIF micelles. By a pharmaceutical point of view, these results indicate that the extemporary reconstitution of lyophilized RIF-loaded systems could give stable suspensions up to, at least 30 days. It implicates that such formulations could be used as a multi-dose pharmaceutical preparation avoiding the costs related to mono-dose formulations. In another study we demonstrated the size stability of INVITESA micelles even in PBS pH 7.4 and at pH 1.2 up to 30 days [16].

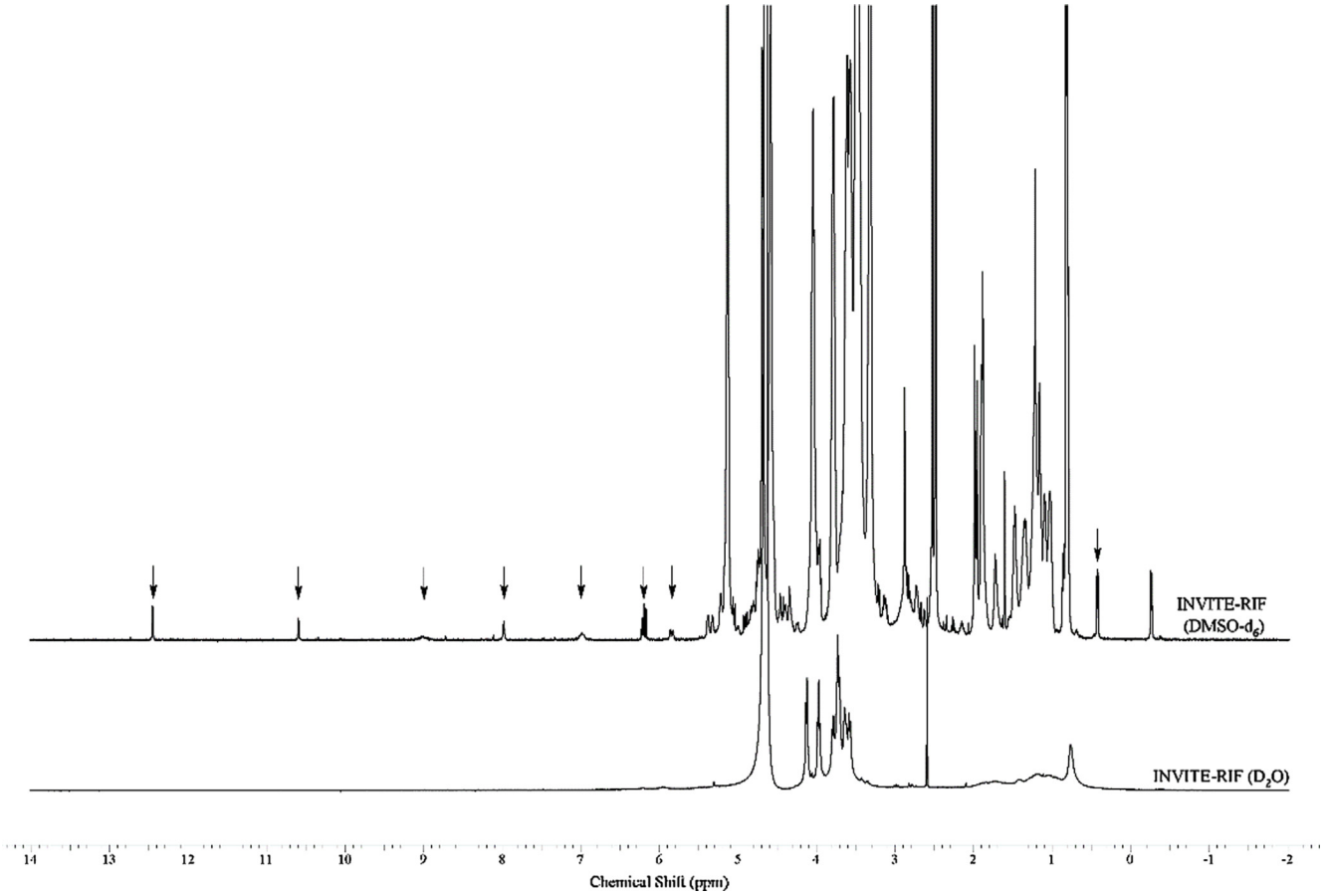

Fig. 1. ^{1}H NMR study showing the disappearance of characteristic RIF peaks when loaded into INVITE micelles in water, the arrows indicate RIF peaks in DMSO-d_6.

3.1.2. Mucoadhesion studies

Mammalian epithelia are protected by a mucus layer mostly characterized by the presence of mucin. The family of mucins is belonging to a wide class of high-molecular-mass glycoconjugates with several oligosaccharide chains in O-glycosidic linkages to a protein backbone. In particular, in the airways, the secreted mucins, are synthesized in specialized cells in the surface epithelium (goblet cells) and submucosal glands (mucous cells). [25,26] The presence of mucins is strongly increased in infectious and inflammation conditions. The presence of specific substituents on the surface of, e.g., polymeric micelles or nanoparticles, such as carboxyl or hydroxyl, could increase, if not attribute, bioadhesive properties to a so structured drug delivery systems [27]. Thus, aimed at verifying the mucoadhesion properties of INVITE and INVITESA micelles, mucin-micelles interaction studies were performed by a turbidimetric assay [28]. This well-established method is conceptually based on the fact that a substance interacting with mucin will determine the formation of macro-aggregates that will reduce the transmittance of a suspension with respect to the pure mucin dispersion. It is also known, that molecular weight plays a fundamental role in determining the magnitude of the mucoadhesive effect, for these reason, in this study we used as a positive control a well-known mucoadhesive polymer such as polyacrylic acid at a molecular weight close to our polymers (5000 Da), the results of which are shown in Fig. 3 [29].

The gained results on the interaction between mucin and empty or drug loaded INVITE and INVITESA, indicate that the micelles show mucoadhesive properties comparable to those of polyacrylic acid. In particular, the transmittance value for INVITESA and INVITESA-RIF, was lower with respect to polyacrylic acid and INVITE or INVITE-RIF samples, especially within the first hours of the experiment, thus indicating a higher mucoadhesion with respect to polyacrylic acid. This result indicates that INVITE based micelles could strongly interact with the mucous layer, thereby increasing the residence time of such micelles, e.g., when nebulized in the respiratory tract. The stability of the interaction with mucin has been demonstrated up to 24 h.

3.2. Drug release studies

Drug release studies were further performed to assess whether the entrapped drug was released and to reveal possible differences between

Table 1
Mean hydrodynamic size, polydispersity index and zeta potential of drug loaded or empty INVITE and INVITESA.

Sample	Mean Size (nm) ± SD	PDI[¥]	Zeta potential[*] (mV) ± SD
Unloaded INVITE micelles	7.03 ± 1.1	0.4	− 13.9 ± 0.55
RIF loaded INVITE micelles	8.2 ± 0.98	0.5	−13.4 ± 1.79
Unloaded INVITESA micelles	5.14 ± 1.1	0.45	−21.6 ± 1.5
RIF loaded INVITESA micelles	4.3 ± 0.98	0.7	−14.8 ± 0.8

[*] Inulin zeta potential = −9.14 ± 4.0 mV.
[¥] PDI = polydispersity index.

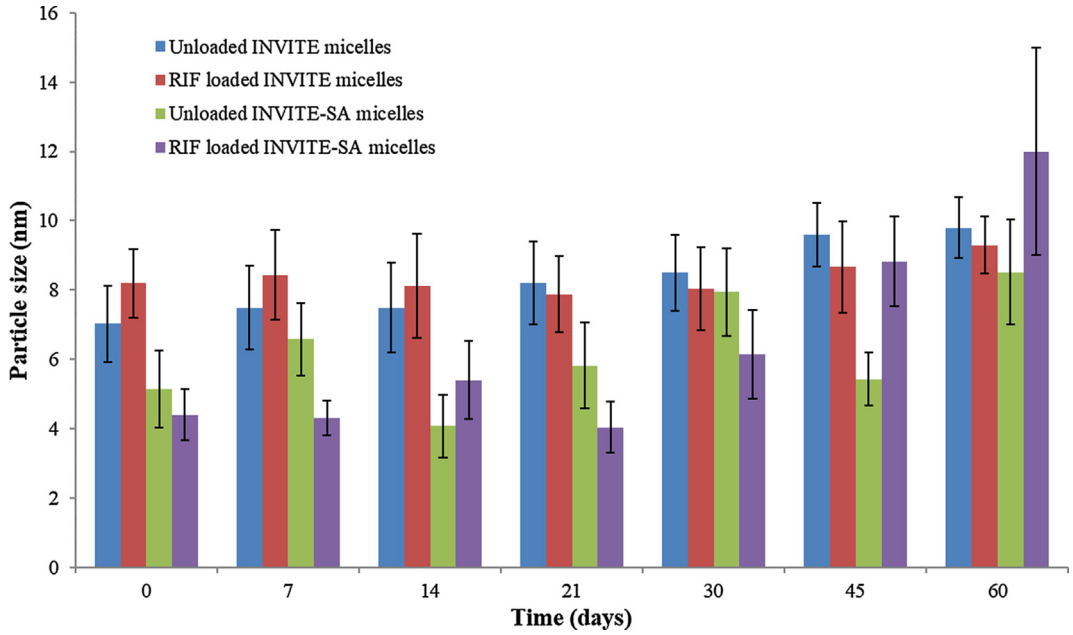

Fig. 2. DLS stability studies on INVITE, INVITESA, INVITE-RIF and INVITESA-RIF micelles in water/NaCl 0.9% w/v at 25 °C up to 60 days.

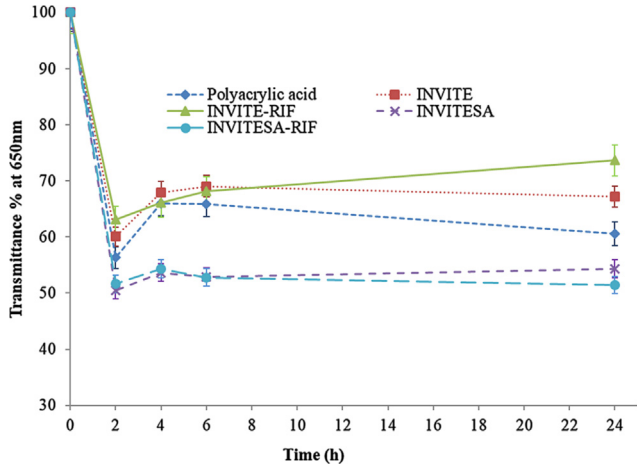

Fig. 3. Mucoadhesion studies performed on mucin for samples INVITE, INVITESA, INVITE-RIF and INVITESA-RIF using polyacrylic acid (Mw 5000 Da) as positive control (mucoadhesive). The reduction in transmittance (increased turbidimetry) indicates a higher interaction polymer-mucins. Mucin dispersion was used as blank during UV measuremnts.

INVITE and INVITESA micelles, Fig. 4.

From Fig. 4, INVITE-RIF or INVITESA-RIF show a comparable drug release profile. In fact, up to seven days both released about 80% w/w of the incorporated drug. The drug release profile is attributable to a classic micellar drug release while no differences could be evidenced between INVITE and INVITESA. Recently, we found that using INVITESA micelles as a pH-sensitive system for the drug delivery of hydrophobic drug into the colon, resulted in a strong difference in the drug release profile at pH 1.2 (stomach) or 6.8 (lower intestinal tract). This result was attributed to a partial degradation of the INU based polymer at pH 1.2 leading to a destabilization of the micelle [16]. In the present work, the INVITE or INVITESA micelles did not undergo any acidic treatment so behaved at the same manner in terms of drug release.

3.3. Evaluation of INVITE-RIF and INVITE-RIF antibacterial activity on selected lung colonizing pathogenic bacteria

The next step was focused on assessing the antibacterial activity of RIF loaded micelles against *Mycobacterium* and against other lung-colonizing bacteria that could cross-affect patients with tuberculosis.

RIF loaded INVITE and INVITESA micelles showed an antibacterial activity comparable to that of free RIF against different pathogenic lung-colonizing bacterial strains. In Table 2 the minimum inhibitory concentration (MIC) values for RIF loaded micelles and free RIF are reported. INVITESA-RIF showed an antibacterial activity comparable to free RIF for all strains. On the other side, INVITE-RIF micelles returned MIC values slightly higher (lower antibacterial activity), in particular, against *S. aureus*, *S. pyogenes*, and *M. smegmatis* than INVITESA-RIF while MIC values for *E. coli* and *P. aeruginosa* were comparable for both INVITE-RIF and INVITESA-RIF. The gained data indicate that, overall, RIF loading into the micelles did not impair its antimicrobial activity. The differences in MIC between INVITE-RIF and INVITESA-RIF could be accounted to the different activity of the different micelles with regard to the Gram positive or Gram negative characteristics of the tested bacteria. As known, Gram positive bacteria expose a peptidoglycan layer to the environment, such layer is composed of alternating residues of β-(1,4) linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). These residues are further including a short peptide chain composed by four to five amino acids such as alanine, glutamine or lysine. INVITESA micelles show, on their external surface, the carboxyl groups from succinic residues that could interact with free amine groups from the peptidoglycan bacterial layer. This would improve the interaction of such micelles with the surface of Gram positive bacteria so increasing the antibacterial activity of these micelles with respect to INVITE micelles. This hypothesis is also corroborated by the fact that INVITE-RIF and INVITESA-RIF both show an antibacterial activity of the same magnitude on Gram negative bacteria [30]. It has been reported that, RIF loaded into nanoparticles based on norbornene-PEG showed a MIC of 0.5 μg/ml, also in the cited study the free and encapsulated RIF did not show significant differences in antibacterial activity [31]. When RIF was loaded into solid lipid nanoparticles (SLN) its antibacterial activity resulted increased [32]. Other authors report that RIF loaded in PLGA NPs inhibited the growth at 70% of the minimum inhibitory concentration (MIC) of pure RIF (MIC level 1 μg/

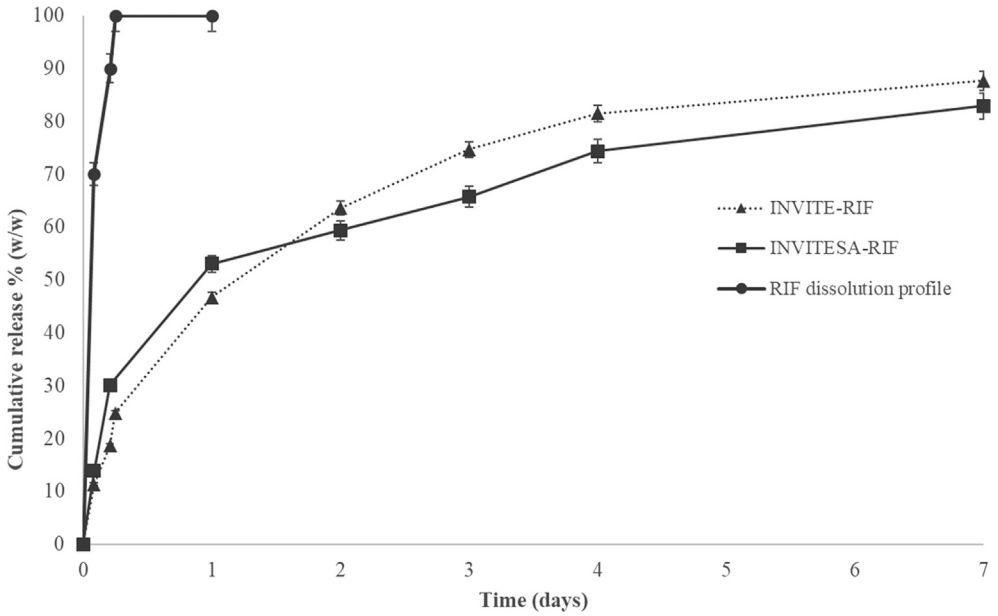

Fig. 4. Drug release studies from INVITE-RIF or INVITESA-RIF micelles in PBS pH 7.4, RIF dissolution curve is further indicated.

Table 2
MIC values of RIF micelles against different lung-colonizing bacterial strains both Gram + or Gram -

Bacterial strains	MIC μg/ml		
	INVITE-RIF*	INVITESA-RIF*	Free RIF
S. aureus (Gram +)	0.18	0.013	0.009
S. pyogenes (Gram +)	13.50	9.00	9.00
M. smegmatis (Gram +)	22.50	18.00	16.00
E. coli (Gram -)	22.50	22.50	18.01
P. aeruginosa (Gram -)	45.00	45.00	34.00

* Empty INVITE or INVITESA micelles did not show any antibacterial activity nor increased bacterial growth.

mL) [33]. Interestingly, RIF loaded into PEG-PLA based micelles showed a MIC value of 0.062 μg/mL against *M. tuberculosis* [34]. It could be expected that the gained antibacterial data for INVITE-RIF and INVITESA-RIF in terms of MIC could be translated to the intracellular (alveolar macrophages) antibacterial activity since it has been demonstrated that the intracellular MIC of RIF is comparable to the extracellular MIC in broth [35].

3.4. INVITE or INVITESA uptake by human alveolar macrophages and cell viability studies by MTT test

To assess whether INVITE-RIF and INVITESA-RIF micelles were taken up by alveolar macrophages, which, as known, are colonized by *mycobacterium* during tuberculosis infection, *in vitro* uptake as well as MTT studies on human macrophages were performed. The human donors were non-healthy subjects. In particular, they are lung-transplanted patients from non-tumor non-infection pathologies. To perform the uptake studies, INVITE and INVITESA were functionalized with FITC.

From Fig. 5 it is possible to note an increase of intracellular fluorescence due to the micelle uptake which showed both time and concentration dependence. INVITESA micelles were taken up less with respect to INVITE, i.e., the intracellular fluorescence was always lower even up to 2 h incubation and at 2 mg/ml. Notably, after 30 min incubation, by increasing the concentration, INVITESA did not show any increase in intracellular fluorescence. On the contrary, INVITE micelles showed a remarkable increase in fluorescence after 2 h incubation as depending from concentration. The increase in intracellular fluorescence indicates a reduced uptake of the micelles by the alveolar macrophages. Similar findings have been described by other Authors; Aijing Lu et al., e.g., reported that micelles composed by poly(ε-caprolactone)-b-poly(N,N-diethylaminoethyl methacrylate)/(N-(3-sulfopropyl-N-methacryloxyethy-N,N-diethylammonium betaine)) (PCL-PDEAPS) or poly (ε-caprolactone)-b-poly(ethylene glycol) (PCL-PEG) are not taken up by macrophages. Only naked PCL-PDEAPS micelles were taken up but, if not adsorbed on plasma proteins, mostly albumin. They also found that the internalization was independent from time and concentration [14]. In this way, we could overcome the plasma proteins binding since inulin does not bind to them, in fact this is one of the most important features of inulin which, as known, also for the lack in plasmatic protein binding, is used to evaluate the renal clearance. In another study, Hsieh-Chih Tsai et al., showed that poly(ethylene glycol)-block-poly(2-methoxyethyl acrylate-co-imidazole) (PEG-b-p(MEA-co-VIm)) micelles were efficiently internalized by HeLa cells while macrophages differentiated between the different shapes of the produced micelles [36]. Yuan Gao et al., produced RIF-loaded micelles from an amphiphilic derivative of hyaluronic acid that showed a concentration dependent uptake by murine macrophages; the proposed micelles had a mean size of 300 nm. In literature has been extensively reported that the factors affecting the nanoparticles uptake by macrophages are several and include size, charge and surface hydrophobicity. As reported in many papers, the nanoparticles size is one of the factors mostly affecting, in general, cell uptake. Even if, it is also reported that this relation size/uptake on phagocytes is very complicated [30,37]. It is generally reported that a size higher than 200 nm could increase the phagocyte uptake [37].

We performed the MTT test of INVITE or INVITESA on the human alveolar macrophages, Fig. 6. The measured cell viability, as cell metabolic activity, is always higher than 60% for both systems with a slight concentration dependence, in particular, for INVITE which viability was above 75% at 0.5 mg/ml while decreased at 63% at 2 mg/ml.

We previously tested several cell lines for their cytocompatibility with INVITE or INVITESA systems. This is the first cell line showing a cytocompatibility lower than 90%. The used macrophages were from non-healthy human donors and were obtained by bronchoalveolar lavage (BAL) from where they were isolated. In any case, a viability above 60% is usually referred to a cytocompatible system. For sure this aspect should be better addressed to evaluate if these results are given

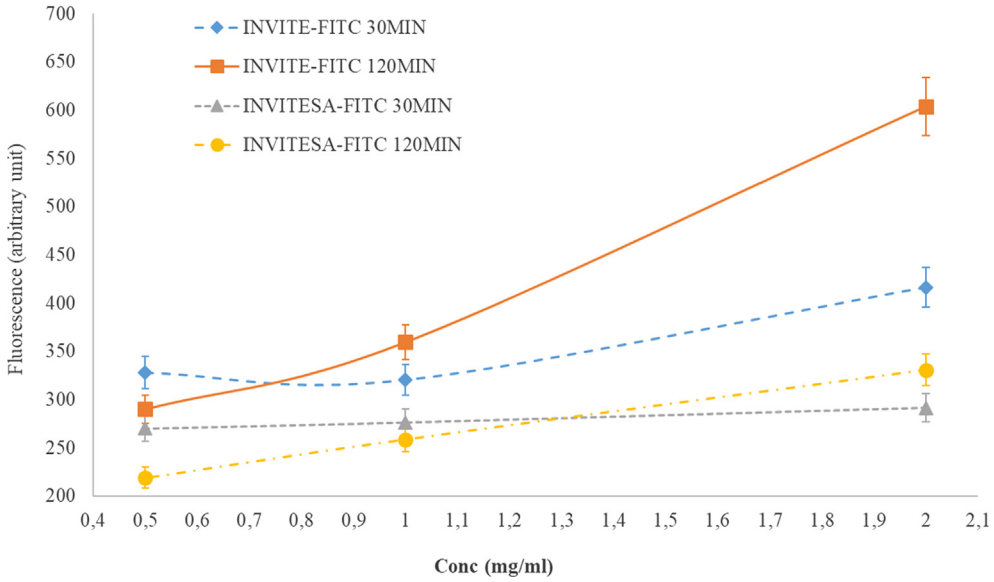

Fig. 5. Uptake studies of INVITE-FITC and INVITESA-FITC on human alveolar macrophages isolated from bronchoalveolar lavage (BAL) as a function of incubation time and micelles concentration.

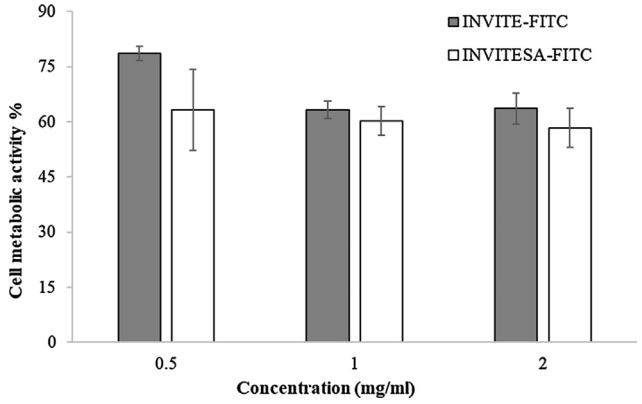

Fig. 6. MTT test of INVITE and INVITESA micelles on human alveolar macrophages isolated from bronchoalveolar lavage (BAL) as direct test after 24 h treatment.

by the kind of used macrophages or to the material itself even if this last assumption, based on our experience, would not be the main explanation.

4. Conclusions

This paper focused on the preparation of a drug delivery system based on two inulin derivatives (namely INVITE and INVITESA) carrying the antituberculosis drug rifampicine (RIF). Two different amphiphilic polymers were synthesized and both were further derivatized with fluorescein for the cellular uptake studies.

We demonstrated that RIF was incorporated within the hydrophobic core of the self-assembling amphiphilic polymers as seen by ^{1}H NMR studies which also allowed to determine the degree of derivatization. Drug loaded or empty micelles resulted nanosized in the order of 10 nm for INVITE and 5 nm for INVITESA. Both systems resulted stable on their size upon storage in aqueous suspension up to 60 days. Furthermore, we demonstrated a strong interaction of such micelles with mucin so predicting favorable mucoadhesive properties.

Then, antibacterial activity of INVITE-RIF or INVITESA-RIF micelles was assessed against mycobacterium and resulted comparable to that of free RIF. In particular, INVITESA-RIF resulted in a higher antibacterial activity against Gram + bacteria with respect to INVITE-RIF. In-vitro INVITE or INVITESA macrophages uptake was also evaluated, showing a moderate uptake of INVITE micelles and a lower one for INVITESA. Cytocompatibility on human alveolar macrophages resulted always higher than 60%.

Considering the obtained results, it is possible to hypothesize the use of INVITE or INVITESA micelles for the drug delivery of hydrophobic antibiotics in the course of lung infections due to *mycobacterium* or other pathogenic bacteria.

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References

[1] P. Glaziou, C. Sismanidis, K. Floyd, M. Raviglione, Global epidemiology of tuberculosis, Cold Spring Harbor Perspect. Med. 5 (2015).
[2] T. Smith, K.A. Wolff, N. Liem, Molecular biology of drug resistance in mycobacterium tuberculosis, Pathogenesis Mycobact. Tuberculosis Its Interaction Host Organism 374 (2014) 53–80.
[3] J. Pieters, Mycobacterium tuberculosis and the macrophage: maintaining a balance, Cell Host Microbe. 3 (2008) 399–407.
[4] M.-H. Xiong, Y. Bao, X.-Z. Yang, Y.-H. Zhu, J. Wang, Delivery of antibiotics with polymeric particles, Adv. Drug Deliv. Rev. 78 (2014) 63–76.
[5] Y. Gao, M.K. Sarfraz, S.-D. Clas, W. Roa, R. Loebenberg, Hyaluronic acid-tocopherol succinate-based self-assembling micelles for targeted delivery of rifampicin to alveolar macrophages, J. Biomed. Nanotechnol. 11 (2015) 1312–1329.
[6] M.A. Moretton, D.A. Chiappetta, A. Sosnik, Cryoprotection-lyophilization and physical stabilization of rifampicin-loaded flower-like polymeric micelles, J. R. Soc. Interf. 9 (2012) 487–502.
[7] M.A. Moretton, R.J. Glisoni, D.A. Chiappetta, A. Sosnik, Molecular implications in the nanoencapsulation of the anti-tuberculosis drug rifampicin within flower-like polymeric micelles, Coll. Surf. B-Biointerf. 79 (2010) 467–479.
[8] J. Morros, B. Levecke, M.R. Infante, Hydrophobically modified inulin from alkenyl succinic anhydride in aqueous media, Carbohydr. Polym. 84 (2011) 1110–1116.
[9] M. Kurecic, M.S. Smole, K. Stana-Kleinschek, Use of polysaccharide based surfactants to stabilize organically modified clay particles aqueous dispersion, Carbohydr. Polym. 94 (2013) 687–694.
[10] L.Z. Zhang, Y. Li, C.P. Wang, G.C. Li, Y.H. Zhao, Y.M. Yang, Synthesis of methylprednisolone loaded ibuprofen modified inulin based nanoparticles and their application for drug delivery, Mater. Sci. Eng. C-Mater. Biolog. Appl. 42 (2014) 111–115.
[11] G. Di Prima, F. Bongiovì, F.S. Palumbo, G. Pitarresi, M. Licciardi, G. Giammona,

Mucoadhesive PEGylated inulin-based self-assembling nanoparticles: In vitro and ex vivo transcorneal permeation enhancement of corticosteroids, J. Drug Delivery Sci. Technol. 49 (2019) 195–208.
[12] M. Licciardi, C. Scialabba, C. Sardo, G. Cavallaro, G. Giammona, Amphiphilic inulin graft co-polymers as self-assembling micelles for doxorubicin delivery, J. Mater. Chem. B 2 (2014) 4262–4271.
[13] L. Catenacci, D. Mandracchia, M. Sorrenti, L. Colombo, M. Serra, G. Tripodo, In-solution structural considerations by H-1 NMR and solid-state thermal properties of inulin-D-alpha-tocopherol succinate (invite) micelles as drug delivery systems for hydrophobic drugs, Macromol. Chem. Phys. 215 (2014) 2084–2096.
[14] A. Lu, Z. Wu, X. Luo, S. Li, Protein adsorption and macrophage uptake of zwitterionic sulfobetaine containing micelles, Coll. Surf. B-Biointerf. 167 (2018) 252–259.
[15] D. Mandracchia, G. Tripodo, A. Latrofa, R. Dorati, Amphiphilic inulin-D-alpha-tocopherol succinate (INVITE) bioconjugates for biomedical applications, Carbohydr. Polym. 103 (2014) 46–54.
[16] D. Mandracchia, A. Trapani, S. Perteghella, M. Sorrenti, L. Catenacci, M.L. Torre, G. Trapani, G. Tripodo, pH-sensitive inulin-based nanomicelles for intestinal site-specific and controlled release of celecoxib, Carbohydr. Polym. 181 (2018) 570–578.
[17] G. Tripodo, G. Pitarresi, G. Cavallaro, F.S. Palumbo, G. Giammona, Controlled release of IgG by novel UV induced polysaccharide/poly(amino acid) hydrogels, Macromol. Biosci. 9 (2009) 393–401.
[18] D. Mandracchia, A. Rosato, A. Trapani, T. Chlapanidas, I.M. Montagner, S. Perteghella, C. Di Franco, M.L. Torre, G. Trapani, G. Tripodo, Design, synthesis and evaluation of biotin decorated inulin-based polymeric micelles as long-circulating nanocarriers for targeted drug delivery, Nanomed.-Nanotechnol. Biol. Med. 13 (2017) 1245–1254.
[19] D. Mandracchia, G. Tripodo, A. Trapani, S. Ruggieri, T. Annese, T. Chlapanidas, G. Trapani, D. Ribatti, Inulin based micelles loaded with curcumin or celecoxib with effective anti-angiogenic activity, Eur. J. Pharm. Sci. 93 (2016) 141–146.
[20] G. Tripodo, G. Pasut, A. Trapani, A. Mero, F.M. Lasorsa, T. Chlapanidas, G. Trapani, D. Mandracchia, Inulin-D-alpha-Tocopherol Succinate (INVITE) nanomicelles as a platform for effective intravenous administration of curcumin, Biomacromolecules 16 (2015) 550–557.
[21] G. Tripodo, T. Chlapanidas, S. Perteghella, B. Vigani, D. Mandracchia, A. Trapani, M. Galuzzi, M.C. Tosca, B. Antonioli, P. Gaetani, M. Marazzi, M.L. Torre, Mesenchymal stromal cells loading curcumin-INVITE-micelles: a drug delivery system for neurodegenerative diseases, Coll. Surf. B-Biointerf. 125 (2015) 300–308.
[22] D. Mandracchia, A. Trapani, S. Perteghella, C. Di Franco, M.L. Torre, E. Calleri, G. Tripodo, A micellar-hydrogel nanogrid from a UV crosslinked inulin derivative for the simultaneous delivery of hydrophobic and hydrophilic drugs, Pharmaceutics 10 (2018) 97.
[23] G. Pitarresi, G. Tripodo, D. Triolo, C. Fiorica, G. Giammona, Inulin vinyl sulfone derivative cross-linked with bis-amino PEG: new materials for biomedical applications, J. Drug Delivery Sci. Technol. 19 (2009) 419–423.
[24] G.A.f.T.D. Development, Rifampin, Tuberculosis, 88 (2008) 151-154.
[25] D.J. Thornton, K. Rousseau, M.A. McGuckin, Structure and function of the polymeric mucins in airways mucus, Annu. Rev. Physiol. 70 (2008) 459–486.
[26] M.C. Rose, Mucins - Structure, function, and role in pulmonary-diseases, Am. J. Physiol. 263 (1992) L413–L429.
[27] N.A. Peppas, Y.B. Huang, Nanoscale technology of mucoadhesive interactions, Adv. Drug Deliv. Rev. 56 (2004) 1675–1687.
[28] G. Pitarresi, G. Tripodo, G. Cavallaro, F.S. Palumbo, G. Giammona, Inulin-iron complexes: a potential treatment of iron deficiency anaemia, Eur. J. Pharm. Biopharm. 68 (2008) 267–276.
[29] F. Thermes, J. Grove, A. Rozier, B. Plazonnet, A. Constancis, C. Bunel, J.P. Vairon, Mucoadhesion of copolymers and mixtures containing polyacrylic-acid, Pharm. Res. 9 (1992) 1563–1567.
[30] S. Xie, Y. Tao, Y. Pan, W. Qu, G. Cheng, L. Huang, D. Chen, X. Wang, Z. Liu, Z. Yuan, Biodegradable nanoparticles for intracellular delivery of antimicrobial agents, J. Control. Release 187 (2014) 101–117.
[31] K. Kumarasingam, M. Vincent, S.R. Mane, R. Shunmugam, S. Sivakumar, K.R.U. Devi, Enhancing antimycobacterial activity of isoniazid and rifampicin incorporated norbornene nanoparticles, Int. J. Mycobacteriol. 7 (2018) 84–88.
[32] E. Aboutaleb, M. Noori, N. Gandomi, F. Atyabi, M.R. Fazeli, H. Jamalifar, R. Dinarvand, Improved antimycobacterial activity of rifampin using solid lipid nanoparticles, Int. Nano Lett. 2 (2012) 33.
[33] S.S. Hakkimane, V.P. Shenoy, S.L. Gaonkar, I. Bairy, B.R. Guru, A ntimycobacterial susceptibility evaluation of rifampicin and isoniazid benz-hydrazone in biodegradable polymeric nanoparticles against Mycobacterium tuberculosis H37Rv strain, Int. J. Nanomed. 13 (2018) 4303–4318.
[34] S. Rani, A. Gothwal, I. Khan, P.K. Pachouri, N. Bhaskar, U.D. Gupta, D.S. Chauhan, U. Gupta, Smartly engineered PEGylated Di-block nanopolymeric micelles: duo delivery of isoniazid and rifampicin against mycobacterium tuberculosis, AAPS PharmSciTech 19 (2018) 3237–3248.
[35] R. Jayaram, S. Gaonkar, P. Kaur, B.L. Suresh, B.N. Mahesh, R. Jayashree, V. Nandi, S. Bharat, R.K. Shandil, E. Kantharaj, V. Balasubramanian, Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis, Antimicrob. Agents Chemother. 47 (2003) 2118–2124.
[36] H.-C. Tsai, C.-H. Chang, Y.-C. Chiu, S.-Y. Lin, C.-P. Lin, G.-H. Hsiue, In vitro evaluation of hexagonal polymeric micelles in macrophage phagocytosis, Macromol. Rapid Commun. 32 (2011) 1442–1446.
[37] S.S. Yu, C.M. Lau, S.N. Thomas, W.G. Jerome, D.J. Maron, J.H. Dickerson, J.A. Hubbell, T.D. Giorgio, Size- and charge-dependent non-specific uptake of PEGylated nanoparticles by macrophages, Int. J. Nanomed. 7 (2012) 799–813.