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Mucopenetration study of solid lipid nanoparticles containing magneto sensitive iron oxide

--Manuscript Draft--

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Abstract:	In most chronic respiratory diseases, excessive viscous airway secretions oppose a formidable permeation barrier to drug delivery systems (DDSs), with a limit to their therapeutic efficacy for the targeting epithelium. Since mucopenetration of DDSs with slippery technology (i.e. PEGylation) has encountered a reduction in the presence of sticky and complex airway secretions, our aim was to evaluate the relevance of magnetic PEGylated Solid Lipid Nanoparticles (mSLNs) for pulling them through chronic obstructive pulmonary disease (COPD) airway secretions. Thus, COPD sputum from outpatient clinic, respiratory secretions aspirated from high (HI) and low (LO) airways of COPD patients in acute respiratory insufficiency, and porcine gastric mucus (PGM) were investigated for their permeability to mSLN particles under a magnetic field. Rheological tests and mSLN adhesion to airway epithelial cells (AECs) were also investigated. The results of mucopenetration show that mSLNs are permeable both in PGM sputum and in COPD, while HI and LO secretions are always impervious. Parallel rheological results show a different elastic property, which can be associated with different mucus mesostructures. Finally, adhesion tests confirm the role of the magnetic field in improving the interaction of SLNs with epithelial cells,. Overall, our results reveal that mesostructure is of paramount importance in determining the mucopenetration of magnetic SLNs,
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Opposed Reviewers:	
Response to Reviewers:	

Dr. Katrien Remaut

Editor

European Journal of Pharmaceutics and Biopharmaceutics

Foggia, 19/07/2022

Dear Dr. Remaut,

please find enclosed the revised version of the manuscript entitled “Mucopenetration study of solid lipid nanoparticles containing magneto sensitive iron oxide”.

We have revised the manuscript accordingly with the Reviewers’ comments. All changes have been highlighted in red when a paragraph was modified or newly added, while periods to be deleted were stricken through.

Please note that a new author was added, i.e. Elvira De Giglio, who performed the XPS analysis on magnetic SLNs.

Sincerely,

Massimo Conese

Reviewer #1: Manuscript Number: EJPB-D-22-00323

Title: Mucopenetration study of solid lipid nanoparticles containing magneto sensitive iron oxide in COPD

General comments: The manuscript reports the results of the mucopenetration evaluation of iron oxide nanoparticles loaded into SLN. The mucopenetration was evaluated using three different kind of mucus (sputum, respiratory secretions aspirated from high (HI) and low (LO) airways, and porcine gastric mucus (PGM)); the adhesion to airway epithelial cells (AECs) were also investigated. The experiments were carried out with rigorous methodology for the aim to understand the mechanisms about particle-mucus interactions and evaluate whether penetration of nanocarriers may be improved under external stimuli.

Instead, it is excessive to conclude or propose nanoparticles for the treatment of the chronic obstructive pulmonary disease. Major revisions are required before the publication in the European Journal of Pharmaceutics and Biopharmaceutics.

Specific comments

1. The Graphical abstract needs to be improved: it is difficult to understand the pictures without reading the manuscript before.

We have completely modified the Graphical abstract to be readily understandable.

2. Pag. 5, Lines 36-41: The sentence: "indicating, in particular, that COPD patients in non-acute clinical conditions are the best target for such application." is not supported by the results. Please modified the sentence.

3. Considering the previous comment, if you believe your claim valid, please add in the Introduction section the current knowledge on the application of IONP in the treatment/diagnosis of chronic obstructive pulmonary disease and their toxicity.

4. In my opinion, "in COPD" in the title could be also removed.

Collectively, we agree with the Reviewer's concerns expressed in points 2, 3 and 4. Thus we have removed the sentence indicated in the point #2 and "in COPD" from the title.

5. The Introduction section is too long: please synthesised!

The Introduction has been synthesised.

6. Pag. 9, Lines 33-55: Please add the correct sub-section titles such as "2.2.1.... and 2.2.2....": it is clearer than a) and b).

It has been done.

7. Pag. 10: Does the release of Liss Rhod PE from SLN has been evaluated? Why Authors assumed that Liss Rhod PE remains on the surface on the basis of interaction as reported in the discussion?

We acknowledge the Reviewer for the issue arised. In the revised version, in Section 2.3, Section 3.1 and in Discussion we have better clarified the interaction occurring between Liss Rhod PE layer and SLNs, demonstrating that no release of the fluorescent tracer takes places in 24h at 37 °C.

8. Pag. 11, Lines 24-27: What means "homogenized by a passage into 10 mL syringe followed

by a further passage through a 2.5 mL syringe". Why you used syringe for homogeniser the sample? Please explain.

We would like to thank the Reviewer for this question. It is known that sputum thickness can make difficult its manipulation. Generally sputum thickness can be reduced through a chemical method, such as mixing of sputum with dithiothreitol (DTT). In our study we could not use this method because DTT is a mucolytic agent that reduces the disulphide bonds present in mucus glycoproteins. Therefore DTT can deeply change the structure of sputum and this could represent an artifact in the study of SLN penetration. For this reason we opted for a mechanical homogenisation in which sputum is passed repeatedly through a 10 ml syringe and then through a 2.5 ml syringe, characterised by a large and tight tip, respectively. These repeated passages through syringes make sputum more manipulable without altering its chemical structure.

9. Pag. 12, lines 44-61: Why 27 h was used as last time point?

Preliminary experiments with both magnetic and non-magnetic fluorescent SLNs at times up to 30 hours (data not shown), showed that the fluorescence recorded in the basolateral compartment in the absence of mucus reached a maximum value at 27 hours followed by a plateau after this time, demonstrating that under these conditions almost 100% of the SLNs passed from the apical to the basolateral compartment in 27 hours. For this reason, 27 hours is the maximum time point used in mucopenetration experiments.

We have added this clarification in Materials and Methods, Section 2.8.

10. Pag. 22, line 28: please remove a "typical" from the caption of Figure 4

It has been done.

11. Pag. 27: The conclusion section is also too long: please revised removing the further discussion and all inserted references and reiterating only the conclusions supported by the results.

It has been done.

Reviewer #2: The paper by Castellani et al. report a mucopenetration study of magnetic solid lipid nanoparticles by using four different type of mucus systems including secretions of high and low airways of COPD under magnetic stimuli. The paper is well organized and written and I recommend it for publication on EJPB after following minor revisions
- authors should explain and comment the rational of IONPs for pulmonary administration in COPD, above all considering the bioelimination of these DDSs that should be very high taking into account the chronic administration normally required in COPD.

We thank the Reviewer for her/his appraisal of our work.

We have now clarified the issue of bioelimination of these DDSs by considering their fast absorption by the capillary bed in the pulmonary circulation and how IONPs may concentrate in the lung upon the application of a magnetic field. All these issues have been included in the first part of the Discussion.

- the structural organization of these IONPs should be expanded by TEM analysis as well as the surface composition by XPS.

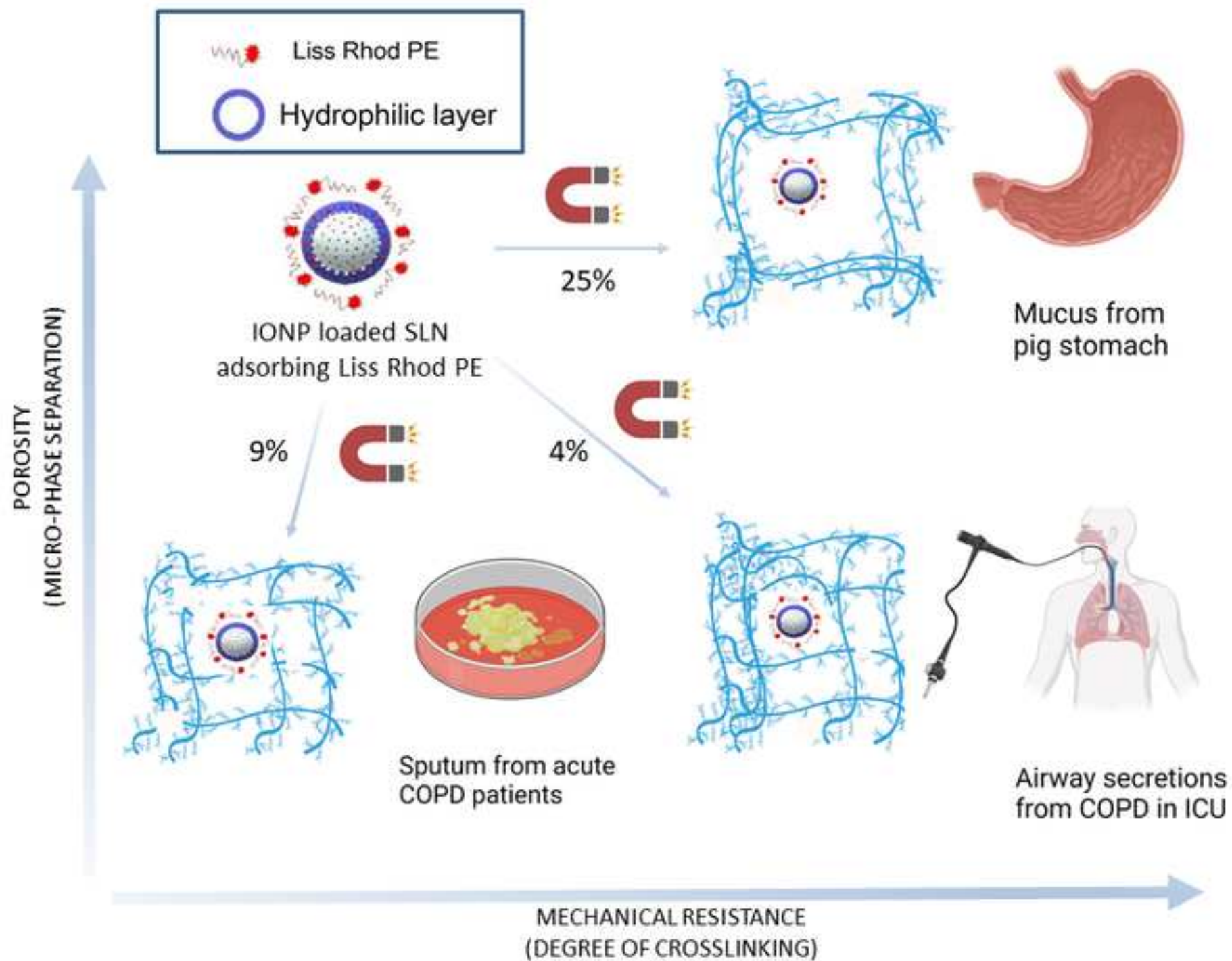
We would like to thank the Reviewer for this consideration on IONPs used in this study, Indeed, we further characterised these IONPs by XPS revealing that some iron was found on the surface of SLNs, a finding that confirms previous results obtained by TEM. We have now included the new M&M, Results and Discussion in the appropriate Sections.

- in the introduction a reference to several platforms based on polymers to overcome the mucus barrier has been done. Authors should expand and discuss these approaches doing some comparison if available.

The appreciation of other platforms based on polymers to overcome the mucus barrier has been expanded in the Introduction.

Highlights

- Sputum and aspirated secretions were obtained from COPD patients
- PEGylated mSLNs penetrated porcine gastric mucus (PGM)>Sputum>Aspirated secretions
- Elastic moduli followed a definite trend: PGM>Aspirated secretions $\geq$ Sputum
- The magnetic field increased mSLNs interaction with primary airway epithelial cells



Mucopenetration study of solid lipid nanoparticles containing magneto sensitive iron oxide in chronic obstructive pulmonary disease

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Abbreviations: AECs (airway epithelial cells); ALI (air-liquid interface); CF (cystic fibrosis); COPD (Chronic Obstructive Pulmonary Disease); DDSs (Drug Delivery Systems); HI and LO (HI/LO) secretions (high and low airway secretions); ICU (intensive care unit); IONP [iron oxide (II, III) NP]; Liss Rhod PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt]; mSLNs (magnetic Solid Lipid Nanoparticles); pAPP (apparent permeability); PGM (porcine gastric mucus); pSLNs (plain Solid Lipid Nanoparticles); TEER (trans-epithelial electrical resistance); **XPS: X-ray photoelectron spectroscopy.**

ABSTRACT

In most chronic respiratory diseases, excessive viscous airway secretions oppose a formidable permeation barrier to drug delivery systems (DDSs), with a limit to their therapeutic efficacy for the targeting epithelium. Since mucopenetration of DDSs with slippery technology (i.e. PEGylation) has encountered a reduction in the presence of sticky and complex airway secretions, our aim was to evaluate the relevance of magnetic PEGylated Solid Lipid Nanoparticles (mSLNs) for pulling them through chronic obstructive pulmonary disease (COPD) airway secretions. Thus, COPD sputum from outpatient clinic, respiratory secretions aspirated from high (HI) and low (LO) airways of COPD patients in acute respiratory insufficiency, and porcine gastric mucus (PGM) were investigated for their permeability to mSLN particles under a magnetic field. Rheological tests and mSLN adhesion to airway epithelial cells (AECs) were also investigated. The results of mucopenetration show that mSLNs are permeable both in PGM sputum and in COPD, while HI and LO secretions are always impervious. Parallel rheological results show a different elastic property, which can be associated with different mucus mesostructures. Finally, adhesion tests confirm the role of the magnetic field in improving the interaction of SLNs with epithelial cells, likely due to both cell surface adhesion and uptake events. Overall, our results reveal that mesostructure is of paramount importance in determining the mucopenetration of magnetic SLNs, indicating, in particular, that COPD patients in non-acute clinical conditions are the best target for such application.

Keywords: Solid lipid nanoparticles; Magnetic field; Rheometry; Mucopenetration; Airway epithelial cells

1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is presently one of the top three causes of death worldwide and its burden is projected to increase in incoming decades because of the continue exposure to COPD risk factors (i.e. cigarette smoking) and aging of the population [1]. COPD is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities. The small airway disease combined with **mucus hypersecretion and** mucociliary dysfunction is a characteristic feature of the disease. ~~The clinical course may be punctuated by periods of acute worsening of respiratory symptoms, called exacerbations, which can range from self-limited diseases to episodes of florid respiratory failure requiring mechanical ventilation [2]. COPD pathogenesis stems from noxious stimuli on the airway and lung epithelia generating the pathologic triad of COPD: persistent inflammation, protease-antiprotease imbalance, and oxidative stress. This triad results in various pathological features, ensuing above all in mucous/goblet cell metaplasia and hyperplasia, and mucous hypersecretion [3].~~

The inhaled route is generally preferred for the delivery of several drugs used in the treatment of COPD. In addition, the topical administration of drugs encapsulated into specific carriers (Drug Delivery Systems, DDSs) has many advantages such as lower doses of drugs to deliver directly to their site of action, causing a rapid onset of action and a low incidence of side-effects. In the case of DDSs through pulmonary airways, however, carriers should overcome the mucus layer to reach the epithelia. In COPD, the sticky expectorated excessive secretions, that are referred to as sputum, oppose a formidable permeation barrier to DDSs to the airway epithelium [2-5]. Indeed, therapeutic efficacy of most colloidal DDSs is often limited because they must overcome numerous biological barriers before arriving at their target sites (cells/tissues). ~~Thus, for instance, a major challenge of many nanostructured DDSs intended for trans-mucosal administration, including oral, respiratory, ocular, vaginal or rectal one, is to overcome the sticky mucus layer which protects the corresponding mucosa from potentially dangerous particles and organisms [8,9]. To bypass this~~

1 hurdle, many efforts have been made in the past by using muco-adhesive nanocarriers, based on the
2 rationale that adhesion of nanocarrier to the mucus should prolong its retention time at the mucosal
3 interface. However, this strategy suffers from the drawback of mucociliary clearance leading to
4 rapid mucus layer turnover and, consequently, only limited amounts of drug are able to penetrate
5 the mucus barrier and to reach the underlying epithelial layers [6]. Hence, interactions of muco-
6 adhesive nanocarriers with the mucus mesh can trap these vectors, thereby preventing their
7 penetration to the underlying tissues. Instead, approaches based on the use of mucus penetrating
8 nanocarriers improve local drug delivery allowing mucus penetration of therapeutic drug amount
9 which can reach so the underlying epithelial layers. Thus, for instance, for obstructive lung diseases,
10 such as COPD, cystic fibrosis (CF), and asthma, it has been demonstrated that benefit resulted to
11 patients from locally delivered drugs to the lung epithelium by penetrating the airway mucus
12 {11,12}.

13 Among the strategies used to facilitate muco-penetration, attention has been focused mainly to
14 those based on the formation of i) slippery surfaces such as those of polyethylene glycol (PEG), (or
15 other hydrophilic polymers)-coated nanoparticles (NPs), ii) virus-like particles exhibiting a high
16 surface charge density but overall neutral, iii) assemblies based on zeta-potential shifting polymers
17 [7, 8]. Moreover, several experimental methods for determining interaction between mucus and NPs
18 including muco-penetration have been developed and recently reviewed [9].

19 In the last decade, many studies have been carried out based on the muco-penetration approach to
20 overcome the mucus barrier from locally delivered drugs. several delivery platforms have been
21 investigated for these purposes being different types of polymeric NPs mainly employed [10]. For
22 instance, mucus-penetrating particles (MPPs) using diblock copolymers of PEG and biodegradable
23 polymers, such as poly(lactic-co-glycolic acid) (PLGA) showed enhanced diffusion rates in cystic
24 fibrosis sputum [11]. Moreover it has been shown that some poloxamers (P103, P105, and F127)
25 with a sufficiently long poly(propylene-oxide) block (≥ 3 kDa) could be absorbed on the PLGA
26 nanoparticle surfaces enhancing the transport of corresponding NPs in human cervicovaginal mucus

[7]. More recently, translational approaches to deliver drugs through pathological mucus included the use of black phosphorus quantum dots (BPQDs) combined with PEGylated chitosan nanospheres to deliver amikacin [12], and graft copolymers based on α,β -poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) for Zileuton [13] or CFTR modulator [14] administration. However, even lipid-based DDSs such as liposomes and solid lipid nanoparticles (SLNs) have been evaluated to develop delivery platforms able to avoid adhesion to mucus layer and to allow its penetration [15-17]. Thus, we have recently reported that 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG 2000PE)-coated liposomes enhanced penetration through COPD mucus, compared with poloxamer 407-coated liposomes and uncoated liposomes [18]. Similarly, mucus-penetrating SLNs, formulated using a mixture of lipids and a poloxamer 407 coating, were prepared to deliver a quorum sensing inhibitor for the treatment of *P. aeruginosa* sustained infections [19].

Another strategy pursued to overcome mucus clearance and guide nanotherapeutics through the mucus barrier, is based on NPs with magnetic cores where magnetic NPs are pulled by external magnetic forces through the mucosal barrier [20-23]. Magnetofection (i.e., the nucleic acid delivery under the influence of a magnetic field acting on nucleic acid vectors that are associated with magnetic (nano)particles) has been used also by our group to improve the dose–response relationship in lentiviral-mediated nucleic acid delivery and in the kinetics of the delivery process, as well as to localize nucleic acid delivery to an area which is under magnetic field influence [24, 25]. In principle, this strategy would reduce the time by which DDSs reside into the mucus/sputum thereby delivering their payload to the airway epithelium with a meaningful efficiency.

As a continuation of our studies in this field, we evaluated whether penetration of colloidal particles through airway sputum may be improved using external stimuli such as those exerted by a magnetic force. To the best of our knowledge, such an issue has been faced down only in few studies using gastrointestinal mucus with exposure to an external magnetic field [23, 26], but, it appears, never using COPD patient expectorated sputum or airway secretions. For the mentioned

1 purpose, we prepared magneto-sensitive iron oxide containing SLNs based on Gelucire® 50/13, a
2 self-emulsifying lipid, considering that they are able to encapsulate with satisfactory efficiency not
3 only lipophilic drugs as occurs with cyclodextrins and polymeric micelles, but also hydrophilic
4 active principles [27, 28]. Moreover, it should be also taken into account that Gelucire® 50/13 is a
5 mixture of PEG-esters (stearyl polyoxyl-32 glycerides), a small glyceride fraction and free PEG
6 chains and, thereby, the resulting SLNs may be considered PEGylated SLNs with a hydrophilic
7 surface [29]. Non-paramagnetic and paramagnetic SLNs, with the above characteristics, were
8 investigated as mucopenetrating DDSs on mucus samples from four different sources: i) a sputum
9 obtained from COPD outpatients, ii-iii) two aspirates from high and low airways of COPD patients,
10 during their admission in the intensive care unit (ICU), iv) a mucus from the pig's stomach. The first
11 three types were chosen for their role in DDS applications, while the PGM was tested as a
12 reference, as it is the “most common ex-vivo model” [30]. Parallel tests to evaluate viscoelastic
13 modules were performed on the four types of samples, along with adhesion test of SLNs to a
14 mucosal surface represented by primary cultures of bronchial epithelial cells.

2. Methods and Patients

2.1. Materials

1 Gelucire® 50/13 was kindly donated by Gattefossè (Milan, Italy). Tween 85, acetic acid, magnetic
2 iron oxide (II, III) NP (IONP) solution were purchased from Sigma Aldrich (Milan, Italy). 1,2-
3 dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt
4 (Liss Rhod PE) was bought from Avanti Polar Lipids. Double distilled water was used throughout
5 the study. All other chemicals were of reagent grade.

2.2. Preparation of IONP loaded SLNs with or without fluorescent coating

2.2.1. Preparation of IONP loaded SLNs without fluorescent coating

For magneto-sensitive iron oxide containing SLN formulation, the melt-emulsification method as previously reported by us [27, 31, 32] was followed. Briefly, 60 mg of Gelucire® 50/13 were melted at 70°C whereas in a separate vial, an aqueous solution (0.37 mL of double distilled water, 1 mL of IONP solution and 60 mg of Tween 85) was incubated at the same temperature. Then, the aqueous phase was added to the melted lipid phase at 70°C in order to obtain an emulsion and homogenization was performed at 12300 rpm for 2 min by the use of UltraTurrax apparatus. After cooling down at room temperature (r.t.) without any ice bath, the resulting SLNs were centrifuged (16,000 × g, 45 min, Eppendorf 5415D, Germany) and the resulting pellet was then freeze dried (LioPascal, Italy).

2.2.2. Preparation of IONP loaded SLNs with fluorescent coating

For fluorescently labelled SLNs, the magneto-sensitive SLNs were produced as above described with the exception that, before to carry out centrifugation, all the nanosuspension was incubated at r.t. for 3 h under light protection with 0.24 mL of the ethanolic solution of Liss Rhod PE at the concentration of 1 mg/mL. The lipid Gelucire® 50/13/Liss Rhod PE weight ratio was set at 0.4% w/w [18]. Once the incubation was over, the resulting fluorescent SLNs were centrifuged (16,000 × g, 45 min, Eppendorf 5415D, Germany) and pellet subjected to lyophilization. As control, plain SLN were produced in the absence of IONP and Liss Rhod PE adsorption, namely for this type of formulation, SLNs were prepared as in a) with the exception that the aqueous phase was composed by 1.37 mL of double distilled water and 60 mg of Tween 85.

2.3. Physicochemical characterization of SLNs

For all tested formulations of SLNs, particle size and polydispersion index (PDI) were measured in double distilled water by photon correlation spectroscopy (PCS) by using a ZetasizerNanoZS (ZEN 3600, Malvern, UK). For particle size analysis, each sample was diluted 1:1 (v.v) with double distilled water [33]. For ζ-potential measurements laser Doppler anemometry technique was

adopted (ZetasizerNanoZS, ZEN 3600, Malvern, UK) and aliquots of SLNs were diluted 1:20 (v.v) with KCl (1 mM, pH 7.0) [34, 35].

Moreover, Liss Rhod PE adsorbed onto SLNs was quantified as follows, calculating the Encapsulation Efficiency percentage (E.E. %):

$$\text{E.E. \%} = \frac{\text{Total amount of Liss Rhod PE} - \text{amount of Liss Rhod PE in the supernatant}}{\text{Total amount of Liss Rhod PE}} \times 100$$

where the total amount of Liss Rhod PE and the mass of Liss Rhod PE in the supernatant were measured fluorimetrically (Varian Cary Eclipse, Mulgrave, Australia, Excitation: 750 nm; Emission 779 nm). For quantitative determinations of Liss Rhod PE, the fluorometer was previously calibrated with standard solutions of from 1 to 140 ng/mL of Liss Rhod PE.

To determine the retention capability of Liss Rhod PE by IONPs loaded SLNs adsorbing Liss Rhod PE, three different NP pellets were split in aliquots and incubated in a water bath (Julabo, Milan, Italy) at 37 °C under stirring (150 rpm) for 24h for appropriate time points (0, 1h, 2h, 4h, 6h, 24h). Once incubation time was over, each aliquot of pellet was centrifuged and the content of Liss Rhod PE in the supernatant was determined fluorimetrically.

X-ray photoelectron spectroscopy (XPS) analysis has been carried out to gain information on the surface chemical composition of the IONPs loaded SLN. The analysis was performed using a scanning microprobe PHI 5000 VersaProbe II (Physical Electronics, Chanhassen, MN, USA), equipped with a monochromatized AlK α X-ray radiation source. The samples were analyzed in HP mode (scanned size: 1400 x 200 μ m, X-ray take-off angle of 45°). For each sample, survey scans (pass energy 117.4 eV) and high-resolution spectra (pass energy 29.35 eV) were acquired in FAT mode. The interpretation of the results by means of a curve-fitting procedure was carried out using MultiPak software package (version 9.9.0.8). The peak areas were normalized by correction with empirically derived sensitivity factors, according to MultiPak library, thus enabling comparison of data from different elements.

2.4. COPD patients

Outpatients clinic in acute phase (n=10) were recruited from the Respiratory Disease Unit (Hospital D'Avanzo, Foggia, Italy) and intubated ICU patients with increasing respiratory secretions (n=7) from the Intensive Care Unit (ICU, Anesthesiology and Resuscitation, Hospital "Riuniti", Foggia). All patients signed the informed consent. The study was approved by the Local Ethical Committee of the Ospedali Riuniti – University of Foggia (n. 83/CE 2019).

2.5. Patients' sample processing

2.5.1. Sputum from outpatient clinic

Outclinic patients were asked to expectorate in a sterile Petri dish. Forceps were used to pull the sputum out of the surrounding saliva [36]. Three samples were discarded since they were mainly composed of saliva. The other seven samples were mechanically homogenized by a passage into 10 mL syringe followed by a further passage through a 2.5 mL syringe. Finally, all samples were pooled and used for experiments.

2.5.2. Respiratory secretions from ICU patients.

Oropharyngeal secretions were obtained before the elective bronchoscopy while bronchial secretions were collected during the elective bronchoscopy by sterile catheters in sterile tubes. The samples were immediately stored at -80°C. No saline was used during the aspiration procedure. Severe traumatic brain injury patients who have anamnesis of COPD according to the British Thoracic Society guidelines were included [37], whereas patients aged under 18, in treatment with inhaled or oral steroids or anti-inflammatory, were excluded.

2.6. Porcine gastric mucus

Porcine mucus samples were taken from the stomachs of freshly slaughtered animals. To collect the mucus layer closest to the epithelium, the internal surface of the stomach is first abundantly washed

with water and then gently scraped with a microscope slide. Sodium azide (weight concentration 0.02% [w/w]) was then added to the collected samples to prevent bacterial growth. The pH of the samples was measured to be between 5.8 ± 0.5 , while their dry fraction ranged from 9 to 13%.

2.7. Rheometric measurements

The rheology of the different mucus was characterized using a Mars III rheometer (Haake), equipped with a 35 mm parallel plates tool. A temperature of 25°C is used in all tests, while the gap in between the plates was approximately 0.8 mm. To prevent water evaporation, silicon oil was placed around the circumferential gap surface. In frequency sweep tests, G' and G'' were always determined in the regime of linear viscoelasticity. Such a regime has been previously assessed by amplitude sweep test at 1Hz. Based on these amplitude measurements a value of $\gamma = 0.01$ has been chosen.

2.8. Study of the mucopenetrating properties of magnetic SLNs

Sputum diffusion studies were performed according to previously published works [18, 38]. One-hundred μL of pooled mucus/sputum was stratified on the upper chamber of a semipermeable filter (Transwell, 0.33 cm^2 , porosity $3 \mu\text{m}$), while 600 μL of PBS were placed on the bottom chamber. Either fluorescently-labelled magnetic SLNs (mSLNs) or plain SLNs (pSLNs) diluted in 100 μL PBS at a final concentration of 16 $\mu\text{g/mL}$ were placed onto the mucus/sputum layer, and the transwells were placed on top of a magnetic plate developing an intensity of 1 T (OZ Biosciences, Marseille, France) at 37°C. As control, the experiments were carried on in absence of mucus layer. Calibration curves are shown in Supplementary Figure 1A & B.

Preliminary experiments with both magnetic and non-magnetic fluorescent SLNs at times up to 30 hours (data not shown), demonstrated that the fluorescence recorded in the basolateral compartment in the absence of mucus reached a maximum value at 27 h followed by a plateau after this time, demonstrating that under these conditions almost 100% of the SLNs passed from the apical to the

basolateral compartment in 27 h. Thus, at different time points (30 minutes, 2 h, 4 h, 6 h, 21 h, 24 h, 27 h) 50 μ L were taken from the basolateral compartment, replaced with 50 μ L of fresh PBS, and then transferred in a 96 well plate and the fluorescence was measured by a fluorimeter. The values were corrected by subtracting the fluorescence in basolateral compartment of transwells in presence of mucus and in absence of nanoparticles at the same time points.

The percentage of mucopenetration was obtained by comparing the fluorescence detected when SLNs were placed in presence of mucus with the maximum fluorescence obtained when SLNs were placed in absence of mucus after 27 h.

The apparent permeability (pAPP) was calculated at different time points by using the following equation:

$$P_{app} = (\Delta Q / \Delta t) / A * C_0$$

where ΔQ is the variation of amount of compound detected in the basolateral side (μ g) (obtained by interpolating the fluorescence data in a calibration curve), in a given time interval Δt (s), A is the surface area of the insert (cm^2), C_0 is the initial concentration in the apical compartment ($\mu\text{g/mL}$).

2.9. Primary airway epithelial cell cultures

Primary bronchial epithelial cells were obtained from the Italian Cystic Fibrosis Foundation (<https://www.fibrosicisticaricerca.it/>). Primary cells were cultured under air-liquid interface (ALI) conditions, to turn the epithelial cells into a mucociliary epithelium [39]. Briefly, isolated primary cells were expanded in LHC9/RPMI medium and then seeded in rat tail collagen-coated Transwells 0.4 μ m porosity filter inserts at a density of 100,000 live cells. After 1day LCH9/RPMI medium will be replaced with DMEM/F12 containing 1% penicillin-streptomycin, 50 mg/mL gentamicin, 2% Ultrosor G (Pall BioSeptra, Cergy, France). After 5-6 days, apical medium was removed to induce the ALI condition. Cells were used after 2 weeks in culture, when complete differentiation is usually reached and the transepithelial resistance is around 700–1,200 $\Omega \cdot \text{cm}^2$. Then, trans-epithelial electrical resistance (TEER) was measured with EVOM equipped with STX2 electrodes.

2.10. Immunofluorescence and confocal microscopy

Secreted mucin 2 and mucin 3 were detected by fixing monolayers in 3% paraformaldehyde and 2% sucrose in PBS, and staining them with unconjugated mouse anti-Mucin-2 (clone SC515032, Santa Cruz) or unconjugated mouse anti-Mucin-3 antibodies (clone SC7315, Santa Cruz, Dallas, TX, USA), (dilution 1:100 for 30 min for both antibodies), followed by incubation with TRITC-conjugated goat anti-mouse secondary antibody (dilution 1:100; Sigma-Aldrich). Then cells were washed three times with PBS and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Cells were rinsed three times with 0.2% BSA in PBS, then filters were excised and placed side up on a glass slide and overlaid with a drop of Fluorescent mounting medium (Dako, Milan, Italy) followed by a coverslip. Fluorescent images were collected using ZOE™ Fluorescent Cell Imager (Bio-Rad, Segrate, Italy).

Cilia formation was assessed through immunostaining with anti-acetylated-tubulin FITC conjugate (Sigma Aldrich). Briefly, monolayers were fixed in 3% paraformaldehyde and 2% sucrose in PBS, and staining them with anti-acetylated tubulin FITC conjugate (dilution 1:100, for 30 minutes). After three washes in PBS, filters were excised and placed side up on a glass slide, and overlaid with a drop of Fluorescent mounting medium (Dako). Cells were analyzed by using a Nikon TE2000 microscope coupled to a Radiance 2100 confocal dual-laser scanning microscopy system (Bio-Rad). Specimens were viewed through a 60X oil immersion objective.

2.11. Interaction of mSLNs and pSLNs with primary airway epithelial cells

ALI-cultures were treated with two doses of both mSLNs and pSLNs (0.1 and 1 µg/mL) in presence of magnetofection for 2 h and 4 h. Then the monolayers were washed three times with PBS, fixed in 3% paraformaldehyde and 2% sucrose in PBS and permeabilised with ice-cold Triton HEPES buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100, pH 7.4) for 5 min at room temperature. Cells were incubated with blocking solution (2% BSA and 2%

1 FBS), for 15 min at 37°C, then with fluorescein isothiocyanate (FITC)-conjugated mouse anti-
2 zonula occludens (ZO-1) antibody (dilution 1:50; Zymed Laboratories, San Francisco, CA, USA)
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4 for 30 min at 37 °C, cells were washed three times with PBS and nuclei were counterstained with
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6 DAPI. Fluorescent images were collected using ZOE™ Fluorescent Cell Imager (Bio-Rad, Segrate,
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8 Italy).
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11 12 13 14 2.12. Statistics 15

16 Statistical analyses of the physicochemical properties of SLNs were carried out by Prism v. 5,
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18 GraphPad Software Inc., USA. Data were expressed as mean $\pm$ SD. Multiple comparisons were
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20 based on one-way analysis of variance (ANOVA) with the either Bonferroni's or Tukey's post hoc
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22 test and differences were considered significant when $p < 0.05$.
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29 30 3. Results 31 32

33 3.1. Preparation and characterization of SLNs 34

35 The magneto-sensitive SLNs under investigation were prepared following the melt-emulsification
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37 method previously reported by us [27, 31, 32] and in Table 1 their main physicochemical properties
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39 are shown. As seen, the greatest differences in particle size were found for plain SLNs adsorbing
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41 Liss Rhod PE, which led to a reduction of particle size up to 73 nm compared to the plain SLNs as
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43 control ($p \leq 0.001$). In contrast, incorporation of IONPs induced a significant increase in mean
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45 diameter value ($p \leq 0.001$), providing mean particle size of 313 ± 14 nm. However, once again,
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47 when Liss Rhod PE was adsorbed on IONPs loaded SLNs, a shrinking of mean diameter was
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49 observed (*i.e.*, 289 ± 44 nm) for IONPs loaded SLNs adsorbing Liss Rhod PE, although the
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51 differences were not statistically significant ($p \geq 0.05$). With the exception of the plain SLNs, all
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53 the SLN samples under investigation exhibited wide PDI values, particularly for SLNs adsorbing
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55 Liss Rhod PE leading to the conclusion that, in these cases a broad size distribution occurs.
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Concerning zeta potential values, all SLNs resulted negatively charged in the range from -9.7 ± 0.8 mV to -21.5 ± 0.5 mV, even in the cases in which the magneto-sensitive iron component was incorporated in the Gelucire[®] 50/13 matrix. The zeta potential values observed for IONP loaded SLNs with and without Liss Rhod PE indicate a good colloidal stability of the corresponding SLNs which should prevent their aggregation. As for Liss Rhod PE content by fluorometric measurements, the percentages of E.E. of the dye Liss Rhod PE obtained (Table 1) resulted higher in the presence rather than in the absence of IONPs (*i.e.*, 21.5% vs 15.7%). Both such percentages can be considered appropriate for fluorometric detection purposes, due to the high fluorescence properties of the dye Liss Rhod PE. Interestingly, when IONPs loaded SLNs adsorbing Liss Rhod PE were incubated up to 24h, negligible amounts of Liss Rhod PE were detected in the aqueous medium ($\leq 1\%$).

Table 1. Physicochemical properties of IONP loaded SLNs.

Formulation	Size (nm)	PDI ^a	Zeta Potential (mV)	E.E. ^b (%)
Plain SLNs	141 ± 11	0.28-0.40	-9.7 ± 0.8	-
IONPs loaded SLNs	$313 \pm 14^{**}$	0.29-0.47	-21.5 ± 0.5	-
Plain SLNs adsorbing Liss Rhod PE	$73 \pm 4^{##}$	0.41-0.42	$-12.1 \pm 1.6^{##}$	15.7 ± 0.6
IONPs loaded SLNs adsorbing Liss Rhod PE	$289 \pm 44^{§§}$	0.55-0.62	$-20.9 \pm 0.6^{§§}$	21.5 ± 0.4

^aPDI: Polydispersion index; ^bE.E.: Encapsulation Efficiency.

Results are expressed as Mean $\pm$ S.D. ($n = 6$). IONPs loaded SLNs vs. Plain SLNs: $^{**}p \leq 0.001$; Plain SLNs adsorbing Liss Rhod PE vs. Plain SLNs: $^{##}p \leq 0.001$; IONPs loaded SLNs adsorbing Liss Rhod PE vs. Plain SLNs adsorbing Liss Rhod PE: $^{§§}p \leq 0.001$.

IONP loaded SLNs have also been characterized by XPS analysis. In Table 2 the surface chemical composition recorded on the investigated sample has been reported. In addition to the expected carbon and oxygen signal, iron has been also detected on the surface of the IONP loaded SLNs.

Table 2. Surface elemental composition of IONP loaded SLNs detected by XPS analysis.

Element	Atomic percentages
C1s	83.3
O1s	15.8
Fe2p	0.9

As far as C1s signal is concerned, after an accurate curve fitting, five peak components can be observed, attributable to five different chemical environments (C-H and hydrocarbon contamination at 284.8 eV; $\underline{\text{C}}\text{-C=O}$ at 285.5 eV; C-OH(R) at 286.4 eV; C=O at 287.4 eV and COOH(R) at 288.9 eV), in agreement with what previously observed on unloaded SLN (data not shown) [29]. Oxygen signal and its relevant curve fitting is reported in Figure 1a. In addition to the two peak components at 532.6 and 533.7 eV, relevant to oxygen in carbonyl/carboxyl group and to alcoholic oxygen, detected also on unloaded SLN [40], a component at 530.0 eV ascribed to O-Fe species can be observed. Fe2p iron spectral region is reported in Figure 1b. Binding energies values of Fe2p_{3/2} and Fe2p_{1/2} equal to 710.6 and 724.1 eV, respectively, can be ascribed to the magnetic iron oxide (II, III) NPs used for the sample preparation. These values are in good agreement with those evidenced by Yamashita and Hayes on a Fe₃O₄ standard sample [41].

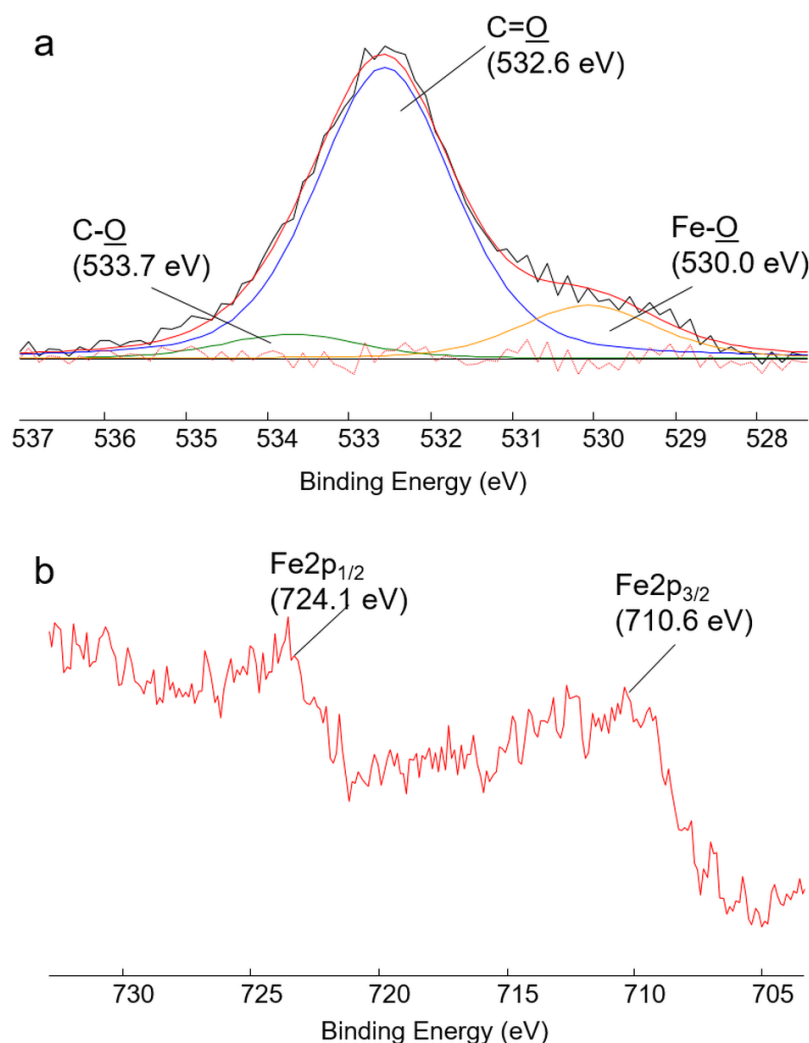


Figure 1. XPS signals relevant to O1s (a) and Fe2p (b) spectral regions. Maximum error on BE values: ± 0.2 eV.

3.2. Characteristics of COPD patients

Outpatient clinic patients were nine males and one female, and the mean age was 77 years (Supplementary Table 1). Eight subjects declared to be ex-smokers, one an actual smoker, and one to have been exposed to asbestos and inorganic powders. Four of them were in oxygen therapy. Based on the FEV1/FVC ratio (Tiffenau index) >0.7 , these patients were in mild condition. CRP

values were indicative of a chronic inflammatory status, witnessed by high numbers of polymorphonuclear cells (Supplementary Table 2).

ICU patients were all males, their mean age was 51 years, and three of them were smokers (Supplementary Table 3).

3.3. Rheological measurements

We tested the different mucus/sputum samples (COPD sputum from outpatients, aspirated secretions from high (HI) and low (LO) airways of COPD patients hospitalized in ICU, and porcine gastric mucus (PGM)) by oscillatory rheology to assess the variations in their viscoelastic properties. To get a statistically significant result, for each type of mucus we performed several frequency-sweep tests. Indeed, it is known that significant variations in the extent of the moduli (G' and G'') occur between specimens belonging to the same type of mucus; whereas, the frequency dependence of such moduli appears to be rather robust [42, 43]. To extract such a dependency, we then normalize $G'(\omega)$ and $G''(\omega)$ of each specimen with respect to the value of G' at the arbitrary frequency of 1Hz. The averages among all the specimens are reported in [Figure 2](#), while the value of G' used to normalize each specimen can be found in Table S4. A gel-like behavior occurs in all cases ($G' > G''$), however, qualitative and quantitative variations are evident between the different mucuses. The porcine mucus, for instance, displays the highest absolute value of the moduli, a dominance of G' over G'' ($G'/G'' > 5$), and a weak frequency dependence. At the opposite, COPD sputum shows the lowest absolute values, a ratio $G'/G'' \approx 2$, and a marked frequency dependence. Intermediate between the two, although closer to the COPD sputum, are the aspirated secretions, which present almost no difference between HI and LO type.

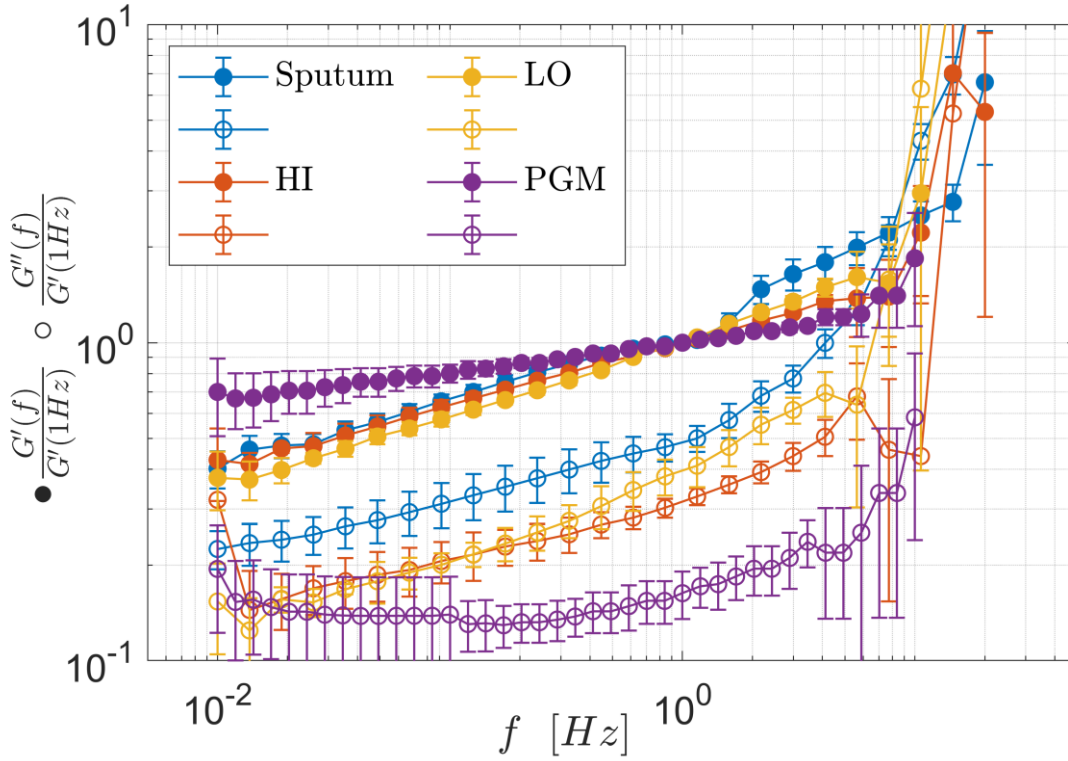


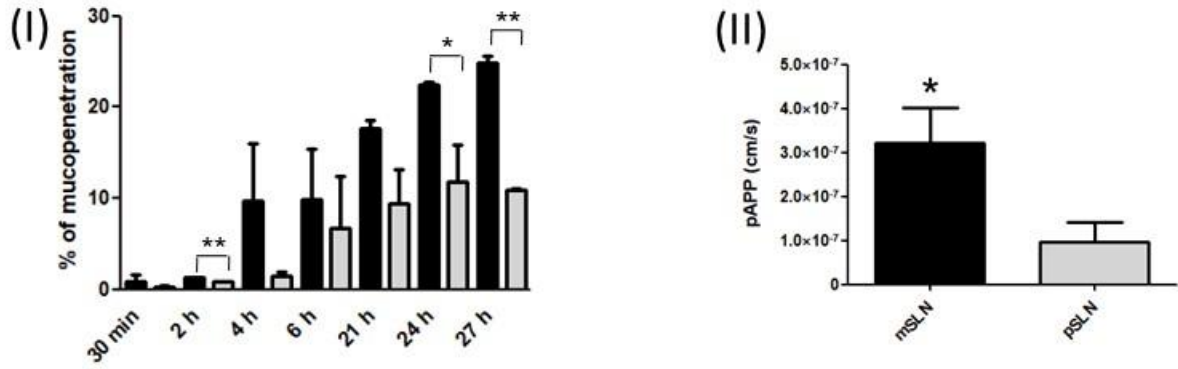
Figure 2. Normalized storage (filled) and loss (empty) moduli vs. frequency, for mucus samples of different sources (see legend). Data are obtained by averaging, after normalization with respect to $G'(f=1\text{Hz})$, at least six frequency-sweep tests in the linear regime ($\gamma=0.01$). The bars indicate the standard deviation over the set of samples. PGM: porcine gastric mucus; HI and LO: high and low airway secretions; Sputum: COPD expectorate.

3.4. Mucopenetration measurements

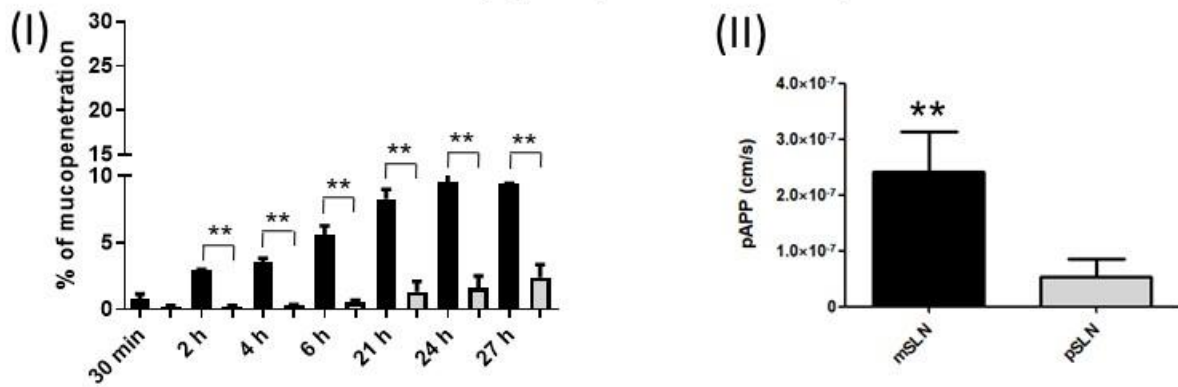
For the sake of clarity, we used fluorescently labelled plain SLNs (pSLNs) and fluorescently labelled IONPs loaded SLNs (mSLNs) in the following studies. In **Figure 3** we report the effect of the magnetic field on the efficiency of mucopenetration of mSLNs and pSLNs particles through the four examined mucus/sputum samples. Unlike the rheological data, the mucopenetration data do not show a clear trend with the sample type. For the PGM the presence of the magnetic field resulted in a more efficient and rapid mucopenetration. Interestingly, the percentage of mucopenetration for pSLNs increased very slowly reaching a maximum value of ~11% compared with pSLN passage in absence of mucus after 27 hours, while we observed a more rapid and efficient penetration for mSLNs with a maximum value of ~25% compared with mSLN passage in absence of mucus after

27 hours (Figure 3a, i). The evaluation of apparent permeability (pAPP) confirmed that mSLNs were significantly more mucopenetrating than pSLNs in the presence of the magnetic field (Figure 3a, ii). When challenged with expectorated sputum in presence of magnetic field, pSLNs and mSLNs demonstrated to be less mucopenetrating in comparison with porcine mucus, reaching ~2% and ~9% of mucopenetration respectively, after 27 hours (Figure 3b, i); interestingly, pAPP was significantly higher for mSLNs in comparison with pSLNs (Figure 3b, ii). When challenged with high and low secretions, mucopenetration slowed down for both pSLNs and mSLNs. Moreover, pAPP measurement reveals that mSLNs were not significantly enhanced in their mucopenetration by the magnetic field in comparison with pSLNs (Figure 3c, i and ii).

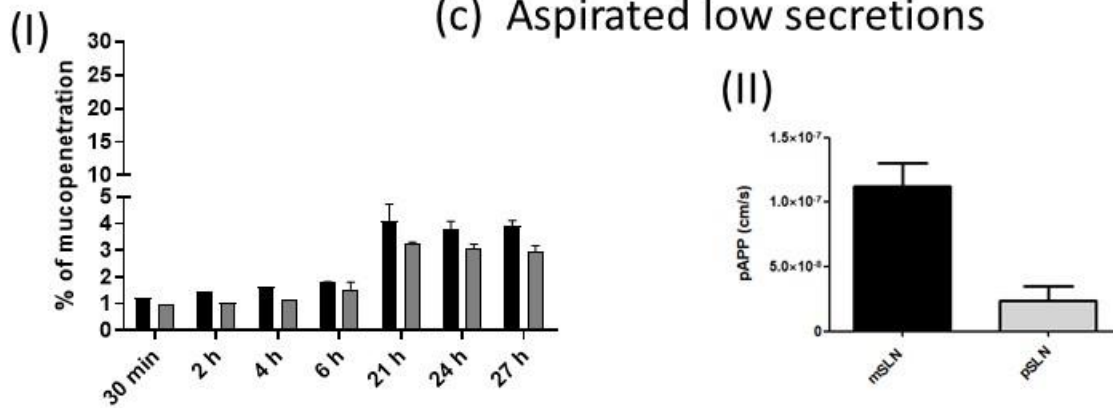
(a) Porcine gastric mucus



(b) Expecterated sputum



(c) Aspirated low secretions



(d) Aspirated high secretions

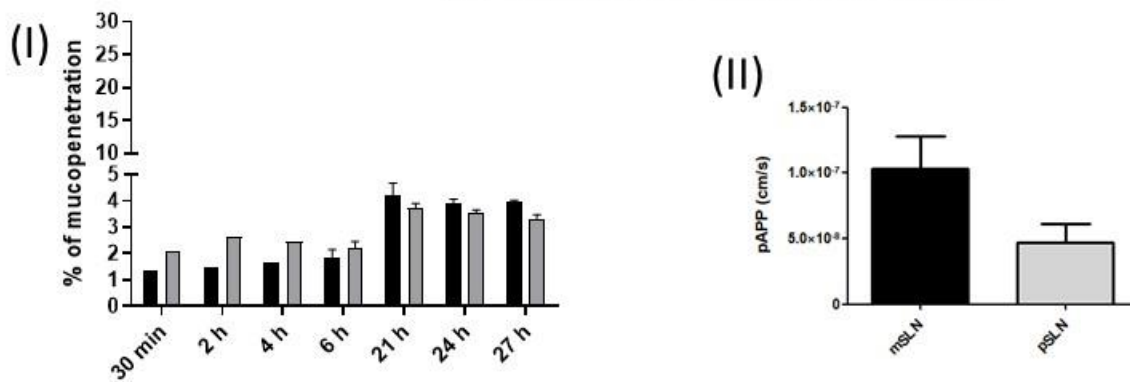


Figure 3. Mucopenetration of mSLNs (black bars) and pSLNs (grey bars). (a) (i) % of mucopenetration through porcine gastric mucus and (ii) pAPP (cm/s). (b) (i) % of mucopenetration through COPD expectorated sputum and (ii) pAPP (cm/s). (c) (i) % of mucopenetration through aspirated high airway secretions and (ii) pAPP (cm/s). (d) (i) % of mucopenetration through aspirated low (ii) airway secretions and (ii) pAPP (cm/s). Data are expressed as mean $\pm$ standard deviation of three experiments. *p < 0.05; **p<0.01 for mSLNs vs pSLNs

3.5. Differentiation into a mucociliary epithelium

After overcoming the mucus layer, DDSs have to interact with the target epithelial cells. To better mimic the in vivo airway epithelium, we have conceived to study these interactions with a differentiated airway epithelium generated from human bronchial epithelial cells. In order to study pSLNs and mSLNs adhesion to differentiated airway epithelial cells in presence of magnetic field, cells were grown on transwells in ALI-conditions for two weeks. Differentiation of primary bronchial epithelial cells in ALI conditions was studied by detecting mucin-2 and mucin-3 and cilia formation by specific antibodies. A mucociliary differentiation occurred at ALI as defined by the detection of both mucin-2 and mucin-3 glycoproteins (Figure 4a) and acetylated tubulin for cilia (Figure 4b).

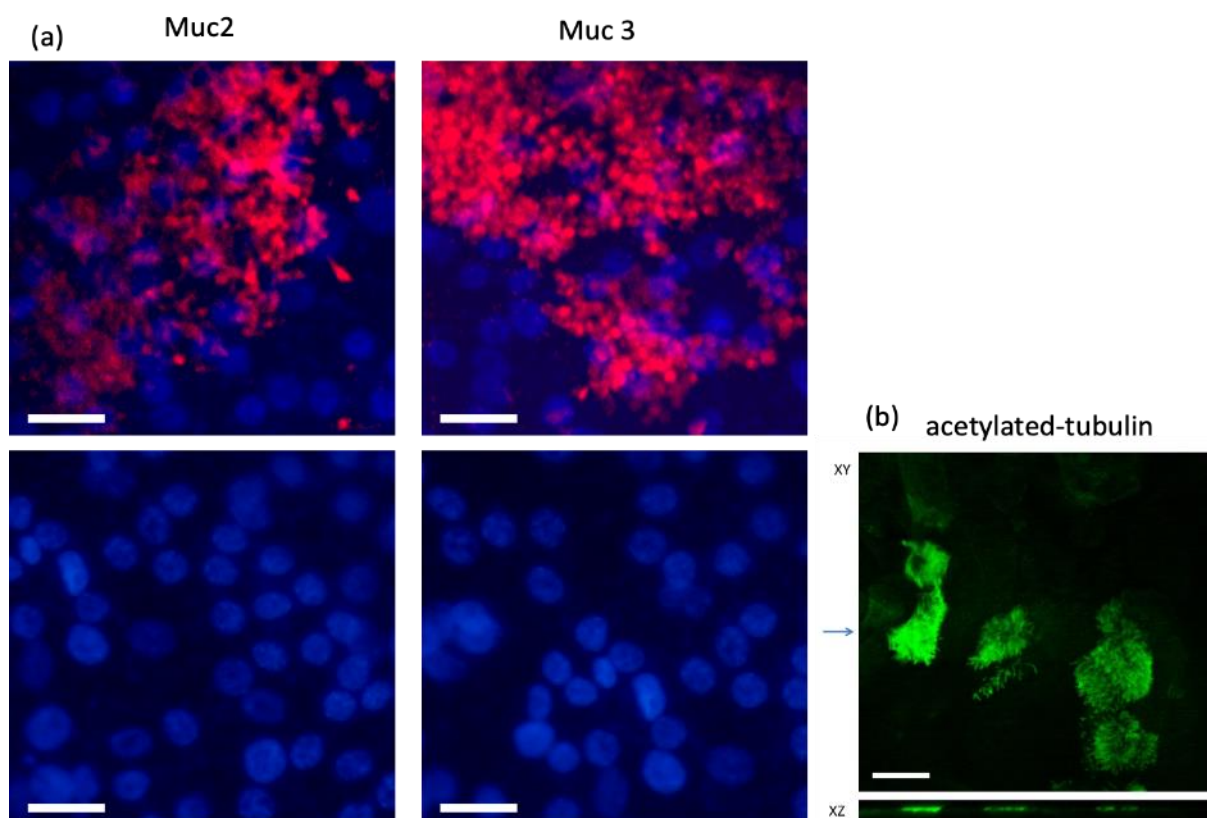


Figure 4. Differentiation into a mucociliary epithelium. (a) Muc2 and Muc3 staining (upper panels); negative controls (lower panels). Scale bar: 10 μm . (b) Acetylated tubulin staining: xy, horizontal section; xz, vertical section. Arrow in xy panel indicates the level of xz reslice. Images were obtained with an original magnification of 60X and after zooming (2.5x). Scale bar: 10 μm .

3.6. Interaction of pSLNs and mSLNs with primary airway epithelial cells

Well differentiated primary ALI cultures were challenged with 0.1 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ mSLNs and pSLNs at 37°C in presence of the magnetic field, and for 2 h and 4 h. Then, cells were fixed and stained for ZO-1 tight junction protein (green signal) while nuclei were counterstained with DAPI (blue signal). mSLNs and pSLNs were detectable in the red fluorescence channel. We observed a dose-dependent rate of adhesion with a strong effect of the magnetic field both after 2 h and 4 h of incubation. Moreover, both pSLNs and mSLNs did not affect the organization of ZO-1 (Figure 5).

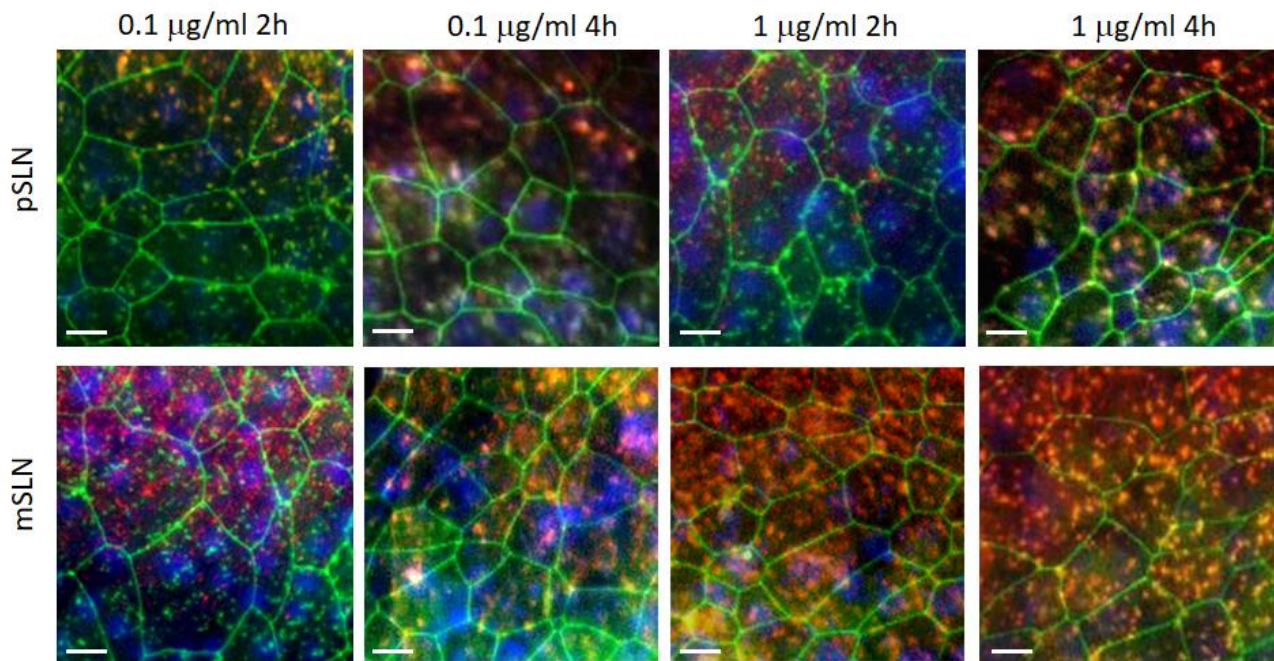


Figure 5. Adhesion of mSLNs and pSLNs to primary airway epithelial cells. After 2 weeks of culture on ALI condition on transwells, airway primary cells were challenged with two different doses of pSLNs and mSLNs in presence of magnetic field for 2 h or 4 h. Cells showed a typical chicken-wire pattern for ZO-1 (green signal). Red signal is associated to SLNs. Nuclei were counterstained with DAPI. Images were obtained with an original magnification of 60X and after zooming (2.5x). Scale bar:10 µm.

4. Discussion

The present work was aimed at evaluating whether penetration of COPD patient sputum/secretions by nanocarriers may be improved under external stimuli such as those exerted by a magnetic force.

Being a chronic disease, COPD would need repeat administration of drugs, even incorporated in NPs. Moreover, inhaled drugs can permeate thin biological barriers, such as the alveoli, and be absorbed into the bloodstream, with a high bioavailability [44-46]. However, magnetic NP, such as those IONPs presented in this paper, are injected directly into the affected site and will be held in place by an external magnetic field to enhance local confinement and release drug in a controlled manner [47]. This issue may be of relevance as for concerns our understanding of the underlying mechanisms about particle-mucus interactions. These last, indeed, still remain to be clarified not only for the drug administration route by airways but also for the oral one, endowed with patient compliance and backing of pharmaceutical industry as well as for other transmucosal administration

1 pathways [30,41,42]. In particular, it could be very important in the challenging case of orally
2 administered nanostructured poor soluble and P-glycoprotein (P-gp) efflux pump substrates (e.g.,
3 anticancer drugs) which are characterized by limited bioavailability [43–45]. For the mentioned
4 purpose, we prepared Gelucire® 50/13-based SLNs for which we suggested, from a structural view
5 point, a hydrophilic shell of PEG chains of solid lipid (Gelucire® 50/13) and co-surfactant (Tween
6 85) together with an internal lipid core represented by the stearyl moieties [29]. With or without
7 Liss Rhod PE, the structural features of IONP loaded SLNs may be conceived supposing that the
8 iron oxide particles are mainly localized in the hydrophilic shell of PEG chains, and for both types
9 of particles herein physicochemical evaluation of size and zeta potential properties have been
10 performed [35]. The interaction of these IONPs with the polyoxyethylene chains of the Gelucire®
11 50/13 improves the colloidal stability as demonstrated by the increase of the zeta potential values.
12 From a dimensional point of view, it is noteworthy the significant reduction of the mean diameters
13 of SLNs obtained from plain SLNs by Liss Rhod PE adsorption (i.e., SLNs adsorbing Liss Rhod
14 PE). This outcome is difficult to rationalize at present time but, in our opinion, it may be accounted
15 for assuming that adsorption of fluorescent dye induces a conformational modification of the PEG
16 chains leading to a shrinking of the size of the resulting SLNs. Actually, IONP loaded SLNs have
17 been also characterized by XPS in order to gain information on their surface chemical composition.
18 XPS analysis evidenced the iron presence on the surface, confirming previous results obtained by
19 transmission electron microscopy (TEM) on the presence of iron both inside and on the surface of
20 paramagnetic SLNs [48, 49], and implying that the mucus penetration of these particles can be
21 actually improved by application of an external magnetic field.

22 Concerning IONP loaded SLNs adsorbing Liss Rhod PE, the interaction between dye and
23 magnetically responsive particles should occur mainly by weak and long range van der Waals
24 forces involving the lipophilic diolyl-stearyl long carbon chains. In addition, also hydrogen bonding
25 interactions between –OH terminal groups of the Gelucire 50/13® molecules and the carbonyl
26 groups of the Liss Rhod PE could be involved. Overall, according to the incubation study described

in Section 2.3, we observed that the adsorption layer made of Liss Rhod PE is quite stable over 24h and less than 1% of dye is lost over the time in the supernatants. Therefore, the risk that the detection of the marker Liss Rhod PE was referred to the dye rather than the entire SLNs was ruled out. COPD, like CF and asthma, is characterized by abnormal mucus deposition into the diseased airways, with its own peculiarities [50] that makes necessary to use pathologic sputum in studies aimed at exploiting gene and drug nanodelivery systems in real life. We thus determined to use different sources of airways mucus from COPD patients, namely sputum and aspirated secretions from high and low airways, to test IONP loaded SLNs mucopenetration capacity. Expectored sputum is composed of lower respiratory tract secretions along with nasopharyngeal and oropharyngeal secretions, cellular debris, and microorganisms [51]. COPD outclinic patients considered in this study were in different GOLD stages, nevertheless their FEV₁/FVC ratio (Tiffenau index) was >75% (i.e. higher of normal values), indicating that these patients were in mild condition [52]. On the other hand, COPD patients requiring mechanical ventilation for severe traumatic brain injury and acute respiratory insufficiency were recruited in ICU. The different resistances encountered by the particles in the mucopenetration (PGM > sputum > HI=LO) were interpreted in terms of structural changes among the different mucus/sputum samples. It is known from the literature [53, 54] that mucus stems from an arrested phase-separation. From this prospective, we can speculate that different mobilities and rheological properties are related to different phase separated structures of the analyzed mucus and secretions. In Figure 5, we report a cartoon showing the envisaged topologies for the examined samples. In the case of porcine mucus, which has the highest elasticity, a more intense phase separation is expected with a consequent more porous structure (Figure 6A). At the opposite, for the elastically weaker sputum and HI/LO secretions, which display also frequency dependent moduli, we expect a higher sol fraction and less “intense” phase separation. In this case, a more “uniform” mesh, characterized by dangling-ends and a corresponding weaker degree of cross-link, should be expected (Figure 6B and C).

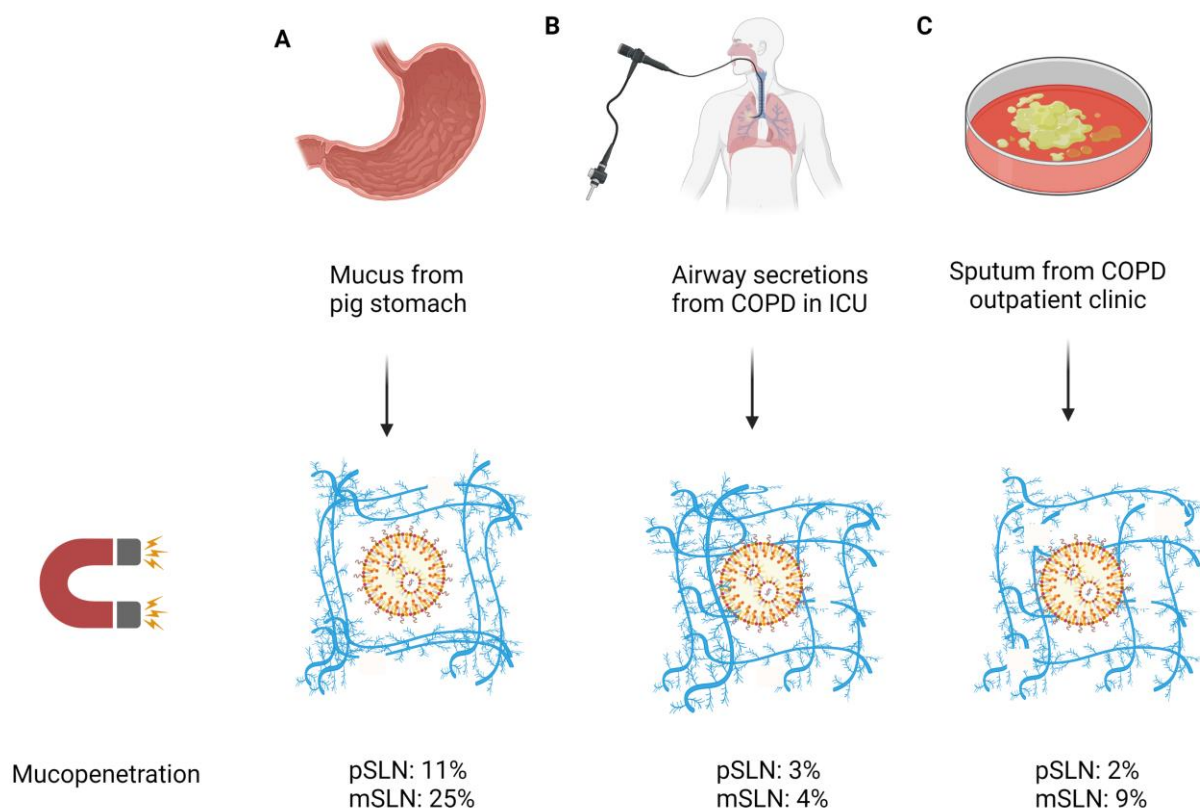


Figure 6. Mucopenetration of mSLNs and pSLNs in the presence of a magnetic field. The hypothetical mesostructure of porcine gastric mucus (A), airway secretions from COPD (B), and COPD sputum (C) is represented. pSLNs penetrate better porcine mucus than COPD sputum or airway secretion. mSLNs penetrate even more porcine mucus while they remain hindered by the airway secretions (either High or Low). However, mSLNs penetrate better COPD sputum than secretions. Created with BioRender.com.

Having the scenario outline above in mind, we can rationalize the different observed permeabilities of non-magnetic and magnetic SLNs as follow. Non-paramagnetic particles are able to diffuse only in the “large” nano-porosity of porcine mucus, while the more “uniform” and viscous mucus types, i.e., sputum and HI/LO secretions, result to be impervious. In the case of paramagnetic particles, the observed behavior varies only marginally. The porcine mucus, already permeable to the SLN particles, shows an even higher permeability to pSLN particles, while the HI and LO secretions, initially impervious, remains so, in line with other works [55]. The only change in particle mobility occurs for the sputum sample. In this case we hypothesize that the force applied by the gradient of the magnetic field is strong enough to overcome the opposite resistance of the

viscous mucus-gel. The net result is a slight increase in the permeability of mSLNs as compared to pSLNs. Such description of our observation is fully supported by the results of Kirch et al. [55], where an impervious mucus from horse bronchi displays the same behavior as our HI/LO samples. The comparison of the average moduli with the one from ref. [55] (see Supplementary Figure 2) leads us to infer that G' in the range of 4-5 Pa is high enough for the elasticity of the mucus to resist the magnetic-diffusive force exerted by the nanoparticle. This is indeed the case for our HI/LO specimens and for the horse bronchial specimens of ref. [55]. On the contrary, for mucus as low as 1 Pa, such as that of sputum, the elastic resistance of the network is overcome by the enhanced mobility of the particles, due to the magnetic field.

Two observations are in order here. First, the elastic spectrum in ref. [55] was obtained from micro-rheological measurements, while our values are instead from macro-rheological measurements. We therefore expect a mismatch of the two values, with the micro-rheology slightly lower than the macro one [54]. Second, in comparing our elastic moduli with the elastic modulus from ref. [55], the latter was averaged considering the values measured at 10 Hz, while our normalized values are taken at 1 Hz. Although we are aware that the elastic modulus increases with frequency, we have chosen these values since a large error occurs in micro-rheology at frequencies lower than 10 Hz, probably induced by insufficient statistics. These observations lead us to consider the proposed comparison with caution, since two counteracting errors are at play. From a qualitative point of view, however, we can assume that the errors due to the different frequency examined and the different technique used tend to compensate each other, making the proposed comparison plausible. We can then state, in line with ref. [55], that “rigid structures within the mucus mesh”, along with porosity, are the basis of our observations.

It is worth noting that to interpret the observed differences in permeabilities we have only invoked the steric hindrance and the elasticity of the network, consistently with ref. [55], where no adhesion effects were taken into account. This sounds appropriate to the case at hand since the nanoparticles used are all PEGylated, therefore, they lack in specific interactions with mucus components.

The mucopenetration is one of the process that therapeutic NPs have to face in order to deliver their cargo to the target airways, the other one being the adhesion to airway epithelial cells and subsequent internalization. This is why we have conducted a proof-of-principle study about the interaction of mSLNs with ALI cultures of bronchial epithelial cells. Their differentiation into a polarised mucociliary epithelium is witnessed by the TEER and the expression of mucins as well as of acetylated tubulin. A number of mucin genes are expressed in the airways [40], with MUC2 and MUC3 gene products found in airway epithelia [56]. However, since ALI cultures were washed before processing to detect ZO-1, and fluorescently labelled SLNs were still found associated with cells, our data suggest that SLNs had overcome the endogenously produced secreted and tethered mucins [57]. Moreover, it is possible that we observe the magnetic field forcing an uptake event, although we did not analyze this by confocal microscopy, as it has been done with ALI cultures of Calu-3 cells and PEGylated lipid-polymer hybrid NPs [58]. Our previous study showed that SLNs used in this study can be taken up by bi-dimensional cultures of airway epithelial cells [32]. Finally, in comparison with our results on the landing of SLN and airway epithelial cells, few studies have surprisingly resolved the issue of nanoparticle tracking when impacted on primary cultures of airway epithelium. Economou et al. [20], using a field gradient of 60 ± 3 T/m (2.5 mm above the tip), successfully pulled anti-inflammation drug (i.e., Dex)-coated FeNPs through the 100- μ m thick mucus layer and reached the cell layer made up by ALI-cultured primary normal human tracheobronchial epithelial (NHTE) cells after 1 h. However, particles tended to form clumps and did not coat the cell layer uniformly. Another study confirmed that magnetic NPs that were initially less than 100 nm in size, to aggregate into sizes of approximately 50 μ m when interacted with mucus produced by NHTE cells [59]. Under our experimental conditions, we did not observe aggregates, although we do not have a direct proof of this, thus we can suppose that SLN used in this study may present that uniform PEG coverage that is critical for efficient drug delivery. This supposition is nevertheless corroborated by the stability of Gelucire®-coated SLNs when incubated with simulated lung fluid at 37°C up to 48 h [32].

5. Conclusion

For a nanotherapeutic system to safely pass across the mucus barrier, researchers have modified both NP size and surface properties. The “slippery” technology, i.e. the nanoparticle coating with PEG, can allow NPs to move quickly through mouse cervicovaginal mucus (~100 μ m layer) [60] and enhance NPs penetration fresh undiluted human mucus [58]. However, PEGylation does not make the difference when the mucus barrier properties are dominated by pathology associated proteins, such as the gel-forming mucins (i.e., sputum from CF patients) [55]. Thus, to guide nanotherapeutics through the mucus barrier, NPs with magnetic cores have been employed [21,22,51,59].

However, also mucus properties make a difference. As regards the four mucus samples examined, the tests showed different mucopenetrations and viscoelastic properties. While viscoelastic properties follow a clear trend with PGM>HI-LO $\geq$ sputum, mucopenetrations depend on the nature of the particles. Non-paramagnetic particles are permeable in PGM alone, whereas the three COPD mucus samples (High, Low, and sputum) are impervious. On the other hand, the paramagnetic particles are permeable in both PGM and sputum COPD, albeit with different values of apparent permeability, and impervious in HI-LO COPD. The intriguing mucopenetration and rheological behavior shown by the mucus/sputum samples were interpreted by assuming a different mesoscopic structure of the samples. This microstructure (i.e., the couple elastic-force and porosity) [51] is always present in the mucus as a consequence of the arrested phase separation to which the mucus is subject. In analyzing the efficiency of a DDSs it is therefore always necessary to compare the particles with the microstructure of the mucus.

In conclusion, the results herein presented highlight that paramagnetic SLN can achieve mucopenetration properties in the presence of a magnetic field, depending essentially on the viscoelastic features of the mucus/sputum sample. Thus, based on the clinical situation of COPD patients, either mild (sputum) or severe (aspirates), these data suggest that nano-based DDSs might

1 be more successful in delivering therapeutic drugs to the target airway epithelium when the patients
2 are considered in a milder condition.
3

4 The study carried out so far has allowed to test the permeation of paramagnetic particles on
5
6 endogenously produced mucus produced by non-COPD bronchial epithelial cells. From this point
7
8 of view, an interesting aspect, currently under investigations, is related to the microstructure of the
9
10 endogenous mucus produced by non-COPD and COPD airway epithelial cells and the effect of the
11
12 magnetic field on the mucopenetration and cell interaction of the paramagnetic particles.
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17 18 19 **Acknowledgements**

20
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22
23
24

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29 01/04/2019).
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32

33 34 **Declaration of Competing Interest**

35
36 The authors have no conflict of interest.
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41 42 **Appendix A. Supplementary data**

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44 Supplementary data to this article can be found online.
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48 49 50 **REFERENCES**

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Mucopenetration study of solid lipid nanoparticles containing magneto sensitive iron oxide in chronic obstructive pulmonary disease

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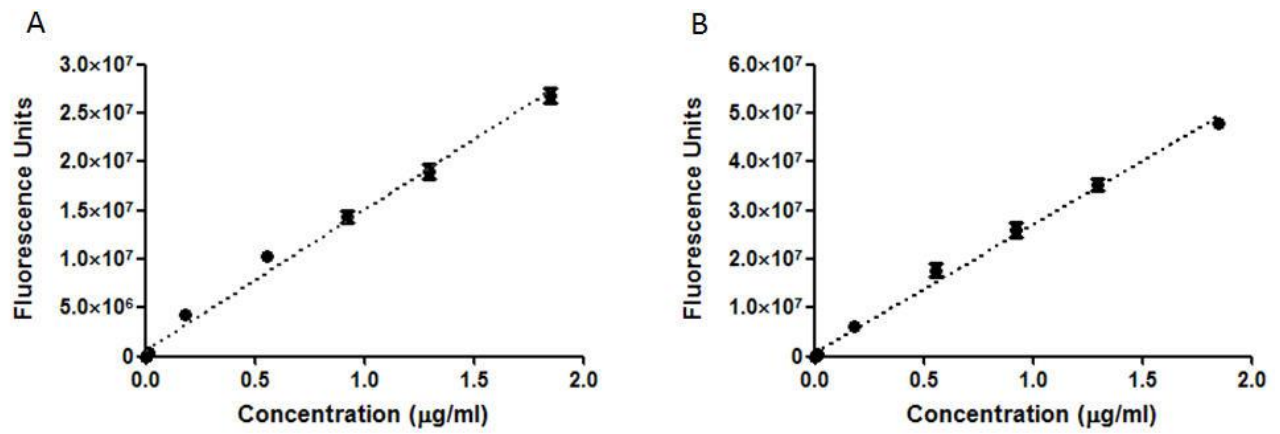


Figure S1. (A) Calibration curve between fluorescent intensity and concentration of magnetic solid lipid nanoparticles and (B) calibration curve between fluorescent intensity and concentration of plain solid lipid nanoparticles. Data are shown as a mean $\pm$ standard deviation of two experiments conducted each in duplicate. $R^2=0.99$ for each curve.

Table S1. Demographic data of COPD outpatient clinic.

#	Age	Sex	Smoker status	GOLD stage [1]	Oxygentherapy	Co-morbidities	Drug therapy
1	78	M	Ex-smoker (120 pack/year)	II B	No	Ischemic-hypertensive heart disease; type 2 diabetes; colonstomy following diverticulitis; right upper lobectomy for well differentiated adenocarcinoma	Glycopyrronium Bromide (Seebri Breezhaler)
2	74	M	Ex-smoker (20 pack/year)	IV D	Yes	Hypertensive heart disease; chronic renal failure; pulmonary arterial hypertension	ICS; LABA; LAMA
3	72	F	140 pack/year	II D	No	Thyroid nodule with euthyroidism; respiratory failure under physical exercise (latent)	Spiriva; Aliflus
4	84	M	Ex-smoker (40 pack/year)	III D	Yes	Ischemic-hypertensive heart disease; type 2 diabetes	Ansimar; Spiriva; Aliflus; Acetylcysteine
5	60	M	No	I B	No	Chronic renal failure; bronchiectasis	Fluimucil; Spiriva
6	92	M	Ex-smoker (120 pack/year)	IV B	Yes	Persistent atrial fibrillation; ischemic heart disease; prostatic carcinoma in hormonal therapy	Seretide
7	77	M	Ex-smoker (150 pack/year)	IV D	Yes	Ischemic-hypertensive heart disease; congestive heart failure; type 2 diabetes; hyperdyslipidemia; benign prostatic hypertrophy; thoracic aortic endoprosthesis; abdominal aortic aneurysm	Aliflus; Bretaris; Gennair

8	90	M	Ex-smoker (100 pack/year)	II C	Yes	Ischemic-hypertensive heart disease	Laventair
9	68	M	Ex-smoker (60 pack/year)	II D	No	Ischemic-hypertensive heart disease; pacemaker wearer; type 2 diabetes	Fluimucil
10	75	M	Ex-smoker (60 pack/year)	II B	Yes	Gastrectomy for ulcer	Seretide

ICS: inhaled corticosteroid; LABA: long-acting β 2-agonist (LABA); LAMA: long-acting muscarinic antagonist.

Table S2. Clinical and laboratory data of COPD outpatients clinic.

	Average $\pm$ SEM	Median	Interquartile range
Age	77.0 $\pm$ 3.1	76.0	71.0 – 85.5
CAT [2]	15.9 $\pm$ 2.6	12.0	9.5 – 24.0
MRC [3]	3.3 $\pm$	4.0	2.7 – 4.0
Acute episodes/year	2.4 $\pm$ 0.3	2.0	1.0 – 2.0
FEV1 (% predicted)	63.4 $\pm$ 7.3	61.5	40.2 – 85.5
FVC (% predicted)	81.4 $\pm$ 6.7	81	60.2 – 125.0
FEV1/FVC	0.78 $\pm$ 0.06	0.80	0.58 – 0.95
CRP (mg/L)	2.8 $\pm$ 9.5	10.3	4.9 – 53.0
WBC (n/mm ³)	8,554 $\pm$ 691	8,530	6,675 – 10,500
PMN (% of WBC)	77.1 $\pm$ 3.8	79.0	65.0 – 81.8
Lymphocytes (% of WBC)	14.9 $\pm$ 2.5	14.6	11.2 – 18.7
Monocytes (% of WBC)	5.5 $\pm$ 0.8	5.1	3.5 – 8.1
Eosinophils (% of WBC)	1.0 $\pm$ 0.4	1.3	0.0 – 1.6
Basophils (% of WBC)	0.2 $\pm$ 0.1	0.2	0.0 – 0.2

CAT: COPD Assesment Test; mMRC: Modified British Medical Research Council Questionnaire.

Table S3. Demographic data of COPD patients admitted in ICU.

#	Age	Sex	Smoker status	GOLD stage	Oxygentherapy	Co-morbidities	Drug therapy
1	65	M	No	II	Yes	Oligophrenia	None
2	56	M	No	I	Yes	None	None
3	59	M	Yes (2 pack/year)	II	Yes	Diabetes, arterial hypertension, ischemic heart disease; L2 vertebrae fracture	Bisoprolol; Acetylsalicylic acid; Metformin; Ivabradine; Atorvastatin; Ramipril+ Hydrochlorothiazide
4	56	M	Yes (2 pack/year)	II	Yes	Microcytic anemia	None
5	59	M	No	I	Yes	None	None
6	20	M	Yes (2 pack/year)	I	Yes	None	None
7	41	M	No	I	Yes	Obesity	None

Table S4. Values of the storage modulus evaluated at 1Hz $G'(f=1)$ used to normalize the data related to Figure 1.

PGM	HI secretions	LO secretions	Sputum
17.00	5.84	3.56	2.02
24.18	4.07	4.19	1.72
25.44	3.79	3.88	1.38
28.64	6.21	4.57	1.86
32.28	5.01	4.88	1.98
33.07	5.04	4.91	1.33
43.97			
47.04			
55.61			
96.90			
185.50			

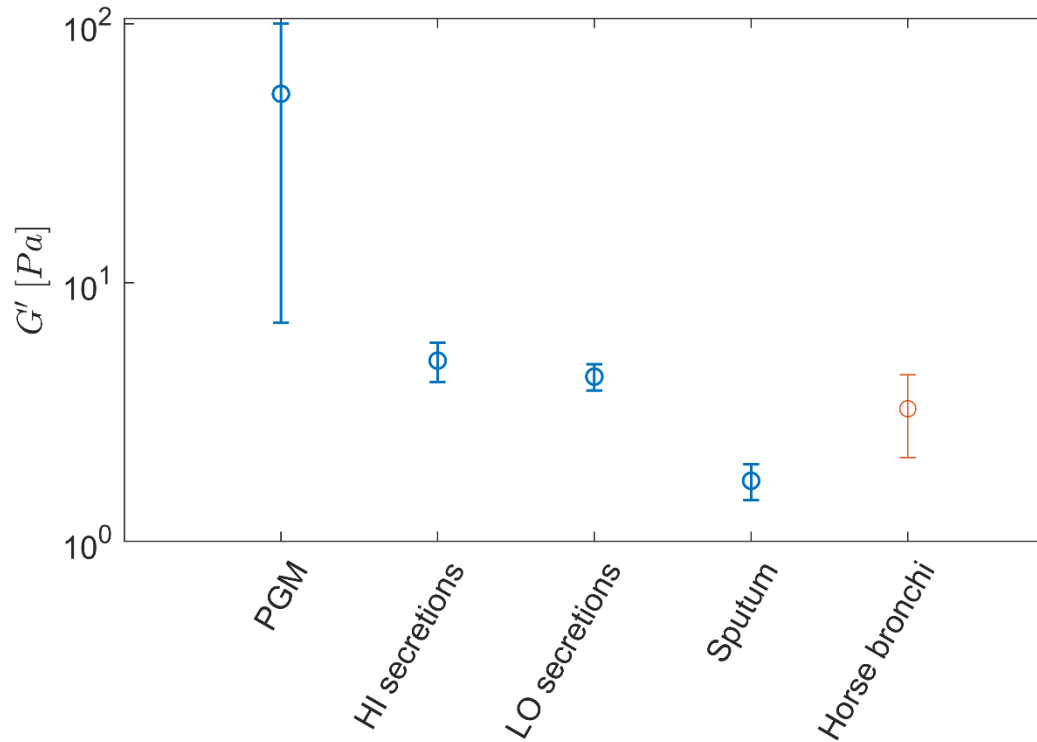


Figure S2. Comparison between the average storage moduli for the different tested mucus with the average modulus of native respiratory mucus from healthy horses [4]. Modulus from ref. [4] has been obtained from micro-rheology, which usually differ from the macro-rheology value by a factor <1 . This discrepancy further strengthens the idea that the HI/LO COPD secretions and bronchial samples of the horse are similar to each other and different from the sputum samples. PGM: porcine gastric mucus; HI: high airways; LO: low airways.

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