

1 *Review*2 **Multifunctional nanocarriers for lung drug delivery**3 **Jorge F. Pontes**^{1,2} and **Ana Grenha**^{1,2,3,*}4 ¹ Centre for Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal;
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12 **Abstract:** Nanocarriers have been increasingly proposed for lung drug delivery applications. The
13 strategy of combining the intrinsic and more general advantages of the nanostructures with
14 specificities that improve the therapeutic outcomes of particular clinical situations is frequent. These
15 include surface engineering of the carriers, by means of alteration of the materials structure (i.e.
16 chemical modifications), the addition of specific ligands so that predefined targets are reached, or
17 even the tuning of the carrier properties to respond to specific stimuli. The devised strategies are
18 mainly directed to three distinct areas of lung drug delivery, encompassing the delivery of proteins
19 and protein-based materials, either for local or systemic application, the delivery of antibiotics and
20 the delivery of anticancer drugs, the latter two comprising local delivery approaches. This review
21 addresses the applications of nanocarriers aimed at lung drug delivery of active biological and
22 pharmaceutical ingredients, focusing with particular interest nanocarriers that exhibit
23 multifunctional properties. A final section addresses the expectations regarding the future use of
24 nanocarriers in the area.

25 **Keywords:** Antibiotics, cancer, drug delivery, lung delivery, nanocarriers, nanopharmaceuticals,
26 proteins.
27

28 **1. Introduction**

29 The appearance of new therapies and alternative strategies for the delivery of drug molecules
30 has been changing the paradigm of therapeutic approaches [1-3]. Indeed, therapeutic solutions have
31 been implemented around one of two possible objectives: one is the development of better and more
32 effective therapies, usually involving new drugs; the other relies on exploring different ways to
33 deliver molecules, potentiating their action and, in many cases, simultaneously, eliminating adverse
34 effects or, at least, decreasing their impact. The latter approach has been often used for drug
35 repurposing, finding new applications for de-risked compounds, with potentially lower overall
36 development costs and shorter development timelines. The literature displays some recent and
37 valuable reviews on the topic of drug repurposing [4,5]. The adverse effects derived from
38 pharmaceuticals have always caused concern, as some can be devastating, leading to therapeutic non-
39 compliance. Thus, exploring delivery strategies is as important as the discovery of new molecules
40 and targets, providing the molecules with specific orientation towards their targets, avoiding major
41 biological stresses and, overall, improving the therapeutic quality [6]. Actually, in many cases, the
42 two referred approaches are addressed at a time.

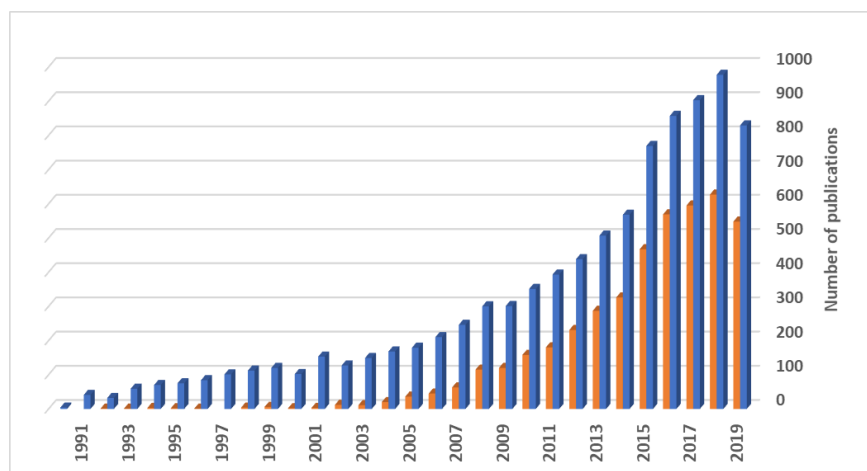
43 Considering that, in most cases, the delivery of unformulated drug molecules is not successful,
44 formulation plays a role of utmost importance in therapy. Conventional drug delivery systems

45 encompass numerous restrictions, which include limited targeting, low therapeutic index, poor
46 aqueous solubility, and the potentiation of drug resistance [7]. The design and production of systems
47 in which drug molecules are included in a carrier, being either embedded in the matrix or adsorbed
48 to the surface, is frequently the next step towards a more effective therapy. The reasons justifying the
49 need for drug formulation are in the annals of pharmaceutical technology, going from a simple
50 protection of drugs to the more complex targeting of cells or tissues. In between, the need to achieve
51 control over drug release has also been a hallmark of drug delivery research. A useful historical
52 perspective on the generations of controlled drug delivery systems is available in [8]. In fact, in an era
53 where drug molecules are expected to answer to increasingly complex environments, their
54 formulation takes on a role never seen. That role assumed, a great variety of advanced drug delivery
55 systems has been reported through the years, with important variations on their properties, including
56 size and composition. Size is one of the most relevant features in the field. In this context, both micro-
57 and nanocarriers have been reported to be viable approaches, the final selection being objectively
58 dependent on the specific application that is envisaged. The potential of micron-sized carriers has
59 been highlighted for different applications [9-13], but lies out of the scope of this review. As for the
60 nanoscaled carriers, in drug delivery these fall into the designation of nanopharmaceuticals, defined
61 by Rivera Gil *et al* as “pharmaceuticals where the nanomaterial plays the pivotal therapeutic role or
62 adds additional functionality to the previous compound” [14]. The International Organization for
63 Standardization defines nanoparticles as those having at least one dimension less than 100 nm [15].
64 In turn, the American Food and Drug Administration (FDA) indicates that products involve
65 nanotechnology, and should therefore be evaluated as such, when they are “engineered to exhibit
66 properties or phenomena attributable to dimensions up to 1000 nm” [16]. This broader definition is
67 the most typically seen in academic research in drug delivery and will be adopted in this review.
68 Therefore, all submicron systems will be considered nanocarriers.

69 As well indicated in the historical description of [8], after an initial period back in the 1980s and
70 1990s, where many micron-sized formulations became popular and reached the market, nanotechnology
71 has been leading the interest of drug delivery scientists since the turn of the new
72 century. In fact, the first mention to a system capable of encapsulating a molecule and providing its
73 transport through a membrane dates back to 1965, under the name of liposome [17], which is, indeed,
74 a nanosystem. From that point on, many other nanoformulations were described and explored, some
75 of them consisting in particulate-based systems, with significant structural differences comparing
76 with the vesicle-based systems comprised by liposomes. Particulate carriers at the nanoscale include
77 polymeric nanoparticles [18], solid lipid nanoparticles [19,20], nanostructured lipid carriers [21,22]
78 and magnetic and silica nanoparticles [23,24]. Probably, issues like increased stability [6], the closer
79 interaction with cell structures [25], the propensity to provide increased drug absorption [26] and the
80 great ability for surface functionalisation [27] have driven the higher popularity of these materials.
81 Regrettably, the clinical translation of nanoparticulate-based systems is very limited so far and only
82 one formulation is available in the market: Abraxane®, marketed since 2005 [28]. This comprises
83 albumin-conjugated paclitaxel, being used in metastatic breast cancer and non-small-cell lung cancer
84 [29]. In this manner, despite the extensive research on particulate-based nanopharmaceuticals, their
85 market absence is notorious, mainly due to tightened regulations. Even so, there are many
86 nanoformulations currently undergoing clinical trials focusing varied routes of administration, and
87 it is expected that some of them make their way into the market in some years [30].

88 From the referred formulations undergoing a translational process and to the knowledge of the
89 authors of this review, none is directed to lung drug delivery. Nevertheless, this is a delivery route
90 that has been gaining popularity in recent years, essentially owing to its non-invasiveness and the
91 increased demonstration of its potential, not only for local therapies, but also to provide systemic
92 action. Actually, according to the World Health Organization (WHO), chronic obstructive pulmonary
93 disease (COPD), lower respiratory infections and lung cancer are, respectively, the third, fourth and
94 sixth causes of death worldwide [31], which illustrates the existing therapeutic limitations. In
95 addition, numerous other respiratory disorders are characterised by an urgent and unmet therapeutic
96 need. The myriad of routes of administration poses the question of which one is the most adequate

97 to deliver a drug intended to treat a specific disease. In parallel, the search for routes of administration
98 other than the oral has been increasing for some years. The lung is now being taken into high
99 consideration for this purpose, as clearly demonstrated in Figure 1, where the number of publications
100 per year that specifically refer to “lung drug delivery”, as retrieved from ISI Web of Science, can be
101 observed. It has been considered a viable alternative in the delivery of drugs and the popularity of
102 this delivery route is reflected in the growing number of publications, especially from 2014 onwards,
103 indicating a clear interest from the scientific community.



119 **Figure 1** – Number of scientific publications under the topics of “lung drug delivery” (blue) and “lung
120 drug delivery and nano” (orange) on ISI Web of Science, as function of the publication year (last
121 updated in January 2020).

122 The established popularity of the lung route relies on several advantages and specific features.
123 Apart from the already mentioned ability to provide either local or systemic effect, characteristics
124 such as high vascularisation and extensive area available for absorption are highly appealing for
125 systemic delivery, while the low metabolic activity compared with the oral route serves both
126 modalities [32]. Furthermore, the possibility to use lower doses and the low incidence of systemic
127 side effects are relevant *pros* for local delivery [33]. A very useful and up-to-date review on the
128 challenges and opportunities of lung delivery can be found in [33]. Despite the mentioned
129 advantages, some limitations are also to be referred, which mainly include the mucociliary clearance
130 as the main mechanism of defence, the patient variability on pathophysiological aspects of the organ
131 and the need to endow the drugs with suitable aerodynamic properties to reach a specific area of the
132 lung [34]. Regarding the latter aspect, the aerodynamic diameter of the drugs or carriers to be
133 delivered through inhalation assumes a crucial role. The aerodynamic diameter is the diameter of a
134 spherical particle with density of 1 g/cm³ and the same settling velocity as the particle of interest. In
135 this context, it is reported that the smaller airways can be reached by particles with aerodynamic
136 diameter lower than 5 µm, while those with less than 2 µm may arrive to the respiratory zone, which
137 includes the alveoli [32].

138 Drugs have been administered by inhalation for millennia, but inhaled therapeutics have been
139 used predominantly to manage common pulmonary diseases like asthma and COPD. In these areas,
140 inhalable drugs have been dominating the market. Systemic formulations, in turn, have been facing
141 many limitations, with significant technical hurdles requiring being addressed before success is
142 achieved. Nevertheless, it has become consensual that, given the offered advantages, the posed
143 challenges are worth addressing. The possibilities have long been debated, especially considering the
144 emergence of biological drugs that are degraded in the gastrointestinal tract and, so, rely uniquely
145 on injection to find efficacy. The scientific community has, thus, been recognising the potential of the
146 lung to be used as a systemic pathway, and many of the papers contributing to Figure 1 deal with
147 systemic lung delivery, although so far this interest is not mirrored by the market. In fact, inhalable

148 insulin is one of the exceptions to mention, appearing first as Exubera[®], from Pfizer (2006), but being
149 discontinued one year after approval [35,36], the company justifying the withdrawal with ‘comercial
150 reasons’. Another product of inhalable insulin became available in 2014, as Afrezza[®], from Mannkind
151 Corporation, and incorporates the Technosphere[®] technology [36,37]. These inhalable insulin
152 products are not based on nanotechnologies, but the scientific community has been recognising the
153 potential of nanocarriers in lung delivery and nanoformulations have been increasingly proposed, as
154 can be also observed in Figure 1. An integrated analysis of this figure shows that publications
155 involving nanosystems usually comprise more than half of the total number of publications on the
156 topic of lung delivery, which demonstrates well their popularity. In fact, the superiority of
157 nanosystems has been demonstrated in certain applications of the respiratory field, as will be
158 described in the following sections of the review. The nanocarriers permit drug protection, provide
159 a greater ability to interact with the tissues and cells, owing to the high surface area, often allowing
160 specific targeting and/or controlled drug release [38]. However, the proposal of nanocarriers must
161 not be blind and it is important to note that some applications may take greater benefit from the use
162 of microcarriers, for example if the therapeutic target is phagocytic cells such as macrophages.
163 Moreover, despite the large amount of works describing nanocarriers for lung delivery applications,
164 it is worth saying that a closer reading of the searched documents reveals that many of the works
165 propose the nanocarriers as having potential from a conceptual point of view, but a much smaller
166 amount approaches the practical concept of preparing the carriers for inhalation, endowing them
167 with the required properties, namely aerodynamic, for the purpose.

168 While initial approaches on the development of drug nanocarriers essentially addressed issues
169 of drug stability and control over the release, these were rapidly replaced or completed with
170 advanced techniques of particle engineering. Thus, the proposal of more complex carriers naturally
171 came along, with particle engineering techniques endowing the nanocarriers with specific properties
172 well beyond their role of carrying a drug or molecule of interest. Such carriers were named as
173 multifunctional and their applications have been explored in all areas of delivery. The
174 multifunctional nanocarrier can be one composed of a material that provides, itself, a specific
175 function, or one that was modified to exhibit a determined feature. Lung delivery can strongly benefit
176 from the features of these carriers. In fact, while most asthma and COPD drugs are delivered to the
177 lung with relatively low efficiency and still ensure therapeutic efficacy, drugs aimed at a systemic
178 action or used to treat orphan diseases or cancer, require optimisation of delivery efficiency. This will
179 render the treatment cost-effective, while potentiating clinical effectiveness and minimising side
180 effects.

181 Considering the interest of nanocarriers within the context of lung drug delivery, this review
182 will focus on their applications, placing particular emphasis on the functionality that is provided by
183 the proper carriers. For the effects of the review, only works addressing directly the issue of
184 pulmonary administration of the carriers, either by adequate *in vitro* testing or by suitable *in vivo*
185 delivery, will be considered, thus going beyond the theoretical concept of suitability for lung delivery
186 purposes. The specific features of the carriers will be referred and the achieved outcomes described.
187 The envisaged applications of the nanocarriers in lung delivery are diverse, but particularly address
188 the delivery of proteins or protein-based materials, either for local or systemic effect [39,40], cancer
189 treatment [41,42] and local delivery of antibiotics [43,44]. Therefore, the review will specifically focus
190 on these topics. As an introductory element to the following sections, Table 1 depicts the major
191 respiratory diseases, along with their main limitations and the potential improvements imparted by
192 pulmonary delivered therapy.
193

194 Table 1 – General overview of the major respiratory diseases, along with their main limitations and improvements imparted by pulmonary delivery of the
 195 drugs. Indication of the application, in each disease, of the drug classes addressed in the review.
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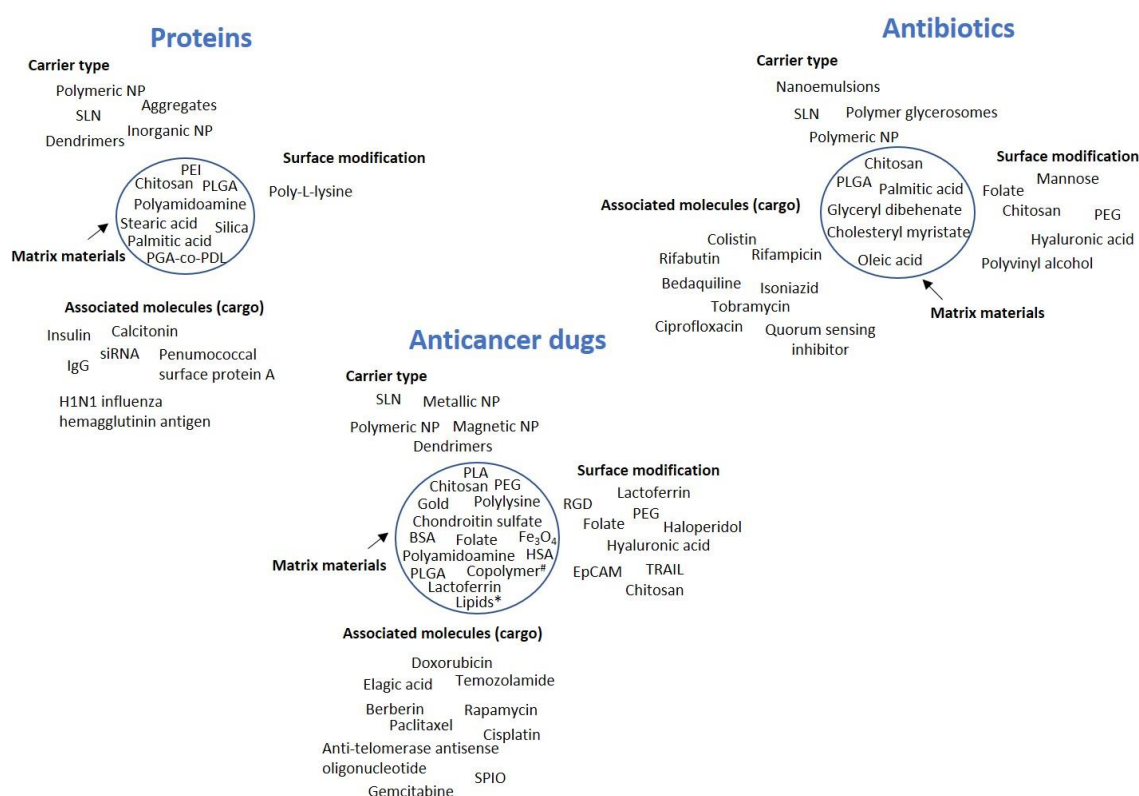
Respiratory disease	Main limitations	Improvements from lung delivery	Proteins	Antibiotics	Anticancer drugs
Asthma	Low therapeutic efficacy of delivered drugs; inefficient control of the disease; airway inflammation	n.a.*	x		
COPD	Persistent inflammation; parenchymal lung tissue destruction; abnormalities of the small airways	n.a.*	x	x	
Pneumonia	Low amount of drug reaches infection site; antimicrobial resistance	Higher drug accumulation in infection site; co-localisation of drug and infectious agent	x	x	
Cystic fibrosis	Thick viscous mucus; recurrent lung infections; progressive impairment of lung airways	Mucus-penetrating carriers; increased lung drug retention; delivery of genetic material to restore CFTR function	x	x	
Tuberculosis	Reduced amount of drug reaches infection site; antimicrobial resistance; long therapeutic regimen	Co-localisation of drug and infectious agent; reduction of antibiotic resistance incidence; possibility of add-on therapy (along with oral); reduce treatment duration		x	
Lung cancer	Non-specificity of drugs; difficulties to reach the affected tissues; severity of systemic adverse effects	Vectorisation to cancer cells; reduction of systemic adverse effects			x

197 CFTR: Cystic fibrosis transmembrane conductance regulator; COPD: chronic obstructive pulmonary disease
 198 n.a.: not applicable; *conventional therapy already administered via inhalation

199 2. Lung drug delivery mediated by multifunctional nanocarriers

200 Multifunctional nanocarriers can be produced from a wide range of materials. In parallel, it is
 201 also wide the number of molecules that can be associated to the carriers to provide specific effects
 202 and improve their performance, either being adsorbed or chemically-bound. This variety arises from
 203 the necessity to meet different challenges and address a vast number of diseases with intrinsic
 204 different characteristics.

205 In the present section, the three main topics mentioned above will be approached. As a summary
 206 of the contents, Figure 2 provides a depiction of the type of carriers used in each topic, along with the
 207 materials selected for nanoparticle matrix and surface modification, when applicable, and also the
 208 associated molecules of interest.



209

210 **Figure 2** – General overview of the types of nanocarriers used in the delivery of proteins,
 211 antibiotics and anticancer drugs, along with the materials applied in the carrier matrix (inside the
 212 circle), the ligands used for surface functionalisation and the associated molecules of interest (the
 213 cargo). The circle indicates the carrier. BSA: bovine serum albumin, EpCAM: epithelial cell adhesion
 214 molecule, HSA: human serum albumin, IgG: immunoglobulin G, NP: nanoparticles, PEG:
 215 polyethylene glycol, PEI: polyamidoamine, PGA-co-PDL: poly(glycerol adipate-co- ω -
 216 pentadecalactone), PLA: polylactic acid, PLGA: polylactic-co-glycolic acid, RGD: tripeptide Arg-Gly-
 217 Asp, siRNA: small interfering RNA, SLN: solid lipid nanoparticles, SPIO: superparamagnetic iron
 218 oxide, TRAIL: tumor necrosis factor-related apoptosis-inducing ligand, #copolymer based on
 219 methoxy poly(ethylene glycol)-poly(ethylenimine)-poly(L-glutamate), *lipids: glyceryl
 220 monostearate, cholesterol.

221 2.1. Delivery of proteins and protein-based materials

222 The marked biotechnological advances observed in recent decades resulted in the appearance
 223 of many protein-based drugs. Fundamentally, the oral delivery of these molecules is prevented by
 224 the degrading effect of abundant protease content and the possibilities of delivery are essentially

reduced to injection-based strategies. However, this approach is more expensive and not appreciated by the patients, mainly due to the discomfort associated with the administration, but also because of some issues related with aesthetics, including bruising and skin marks that may compromise therapeutic compliance [45]. The pulmonary route thus appears as a sound alternative when a systemic effect is desired, but protein-based drugs also find applications in the treatment of local lung diseases. In fact, the first inhaled protein reaching the market was recombinant human DNase (rhDNase), indicated in the treatment of cystic fibrosis and available since the late 90s. Inhalable insulin appeared approximately 10 years after and lessons learnt from its development resulted in the current availability of many elegant inhalation devices and formulations. So far, no other inhaled biological drug aimed at systemic delivery reached the market, despite those being the drugs focusing most of the attention within the context of systemic delivery mediated by inhalation. None of the referred marketed formulations encompasses the use of nanocarriers. Actually, members of our group participated in the first work proposing the inhalation of insulin encapsulated in polymeric nanoparticles, dated back to 2005 [39,46,47]. At that time, chitosan was proposed as matrix material, resulting in non-toxic nanoparticles and endowing the system with mucoadhesivity [48]. In order to provide the nanoparticles with suitable aerodynamic properties to reach the alveolar zone, a nano-in-micro system was developed, using spray-drying to microencapsulate the nanoparticles in mannitol microspheres. These, expectedly released the nanoparticles after dissolving in the lung lining fluid, providing the release of the protein that could, thus, be absorbed systemically [39]. An *in vivo* study in rats evidenced that microencapsulated insulin-loaded chitosan nanoparticles administered intratracheally (IT, 16.7 IU/kg) induced a more pronounced and prolonged hypoglycemic effect compared with insulin solution, as observed in Figure 3 [47], thus demonstrating the contribute of the carrier itself to the observed therapeutic effect. A similar approach was later on proposed by other authors, using poly-L-lysine to modify the surface of self-assembled pure insulin nanoaggregates, benefiting from the adhesive properties of poly-L-lysine. After IT administration to diabetic rats, the modified nanostructures (5 IU/kg) induced hypoglycemic effect as stronger as subcutaneous delivery (1 IU/kg), but increasing the drug half-life from 1.28 h to 2.75 h. Although not statistically significant, the hypoglycemic effect obtained from nanoparticles was also more prolonged, achieving 23.4% relative bioavailability [49].

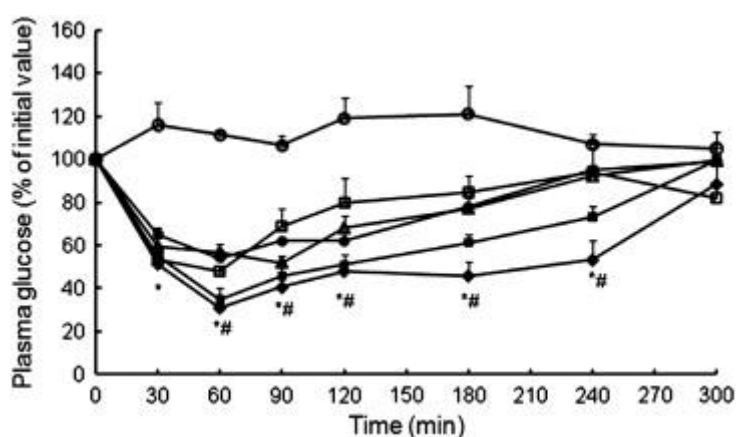


Figure 3 – Hypoglycemic profiles following intratracheal administration to rats of microencapsulated insulin-loaded chitosan nanoparticles (INS-loaded CS NPs) prepared using chitosans of different MW (CS 113 and CS 213), and control formulations (mean \pm SD, $n \geq 3$): (◆) Microencapsulated INS-loaded CS NPs – CS 113; (■) Microencapsulated INS-loaded CS NPs – CS 213; (○) Microencapsulated blank (without insulin) CS NPs – CS 113; (□) Mannitol microspheres containing INS; (△) Suspension of INS-loaded CS NPs – CS 113; (●) INS solution in PBS pH 7.4; *Statistically significant differences from microencapsulated blank CS NPs ($p < 0.05$); #Statistically significant differences from INS solution ($p < 0.05$). Reprinted with permission from [47].

276 Solid lipid nanoparticles (SLN) were also proposed for this end and were reported to provide
277 homogeneous distribution through the lung upon delivery to diabetic rats by nebulisation, showing
278 relative bioavailability of insulin of 22.3% comparing with subcutaneous injection [50]. An approach
279 similar to that referred above of chitosan nanoparticles microencapsulated in mannitol microparticles
280 was later reported for the systemic delivery of calcitonin. The inhalable carriers had mass median
281 aerodynamic diameter (MMAD) of 2.7 μm and fine particle fraction (FPF) of 64%, the latter
282 representing the fraction of particles with aerodynamic diameter lower than 5 μm [51]. After IT
283 administration, around 85% relative bioavailability was determined, comparing with subcutaneous
284 delivery. The bioavailability was also superior to that obtained after inhalation of native calcitonin
285 [52]. Another approach in the same line, proposed the delivery of IgG mediated by poly(lactic-co-
286 glycolide) acid (PLGA)-based nanoparticles produced by double emulsification and subsequently
287 spray-dried to acquire suitable aerodynamic properties. Leucine was further included to improve
288 aerosolisation. MMAD around 4 μm and FPF of approximately 50% indicated suitability to reach the
289 deep lung, while a prolonged release up to 35 days was observed in PBS pH 7.4, enabling applications
290 where prolonged release is envisaged [53].

291 More than simply avoiding injections, the driving force fostering investment on systemic drug
292 delivery through the lung relies on the improvement of pharmacokinetics, which could be an
293 advantage for drugs currently delivered through the oral, buccal or transdermal routes. The studies
294 reported above reinforce the potential of the lung to provide an access to the systemic compartment,
295 but above all, they show that the nanocarriers can play a role in improving the therapeutic
296 performance. Nevertheless, one of the limitations that is relatively transversal to works on lung
297 delivery is the fact that, in most cases, the *in vivo* testing of inhalable formulations is performed by IT
298 administration, which does not mimic the reality when human delivery is concerned. This aspect still
299 requires some advancement in order to better predict *in vivo* outcomes.

300 The delivery of drugs that are specifically directed to the lung, is the other side of the picture of
301 lung delivery. Local treatment of lung diseases usually aims at low systemic bioavailability in order
302 to avoid the risk of unwanted side effects in other organs due to rapid drug translocation via the air-
303 blood barrier. Some respiratory pathologies are ineffectively treated with existing small molecule-
304 based therapies. RNAi effectors, such as small interfering RNA (siRNA), have shown to enable the
305 post-transcriptional silencing of key molecular disease factors that cannot be readily targeted with
306 conventional small molecule drugs [54]. Therefore, some therapeutic alternatives are currently being
307 proposed in this context. The type of cell that is targeted in this approach is variable and depends on
308 the specific airway disease. Epithelial cells are key players in cystic fibrosis, for instance, while
309 dendritic cells, macrophages and T lymphocytes are the targets in inflammatory diseases like asthma
310 or COPD [54]. The local therapeutic response to siRNA can be markedly enhanced through the use
311 of nanoparticles, essentially due to the possibility to provide specific cell targeting. The period of time
312 that siRNA is retained in the lung plays an important role on the success of the approaches. This
313 period is affected by rapid elimination due to mucociliary clearance, translocation to systemic
314 circulation and secondary organs, and phagocytosis by alveolar macrophages. The complexation of
315 siRNA with polyethylenimine (PEI), forming polyplexes, was demonstrated to reduce the
316 translocation and extend siRNA retention time in lung, while preventing substantial phagocytosis by
317 macrophages and avoiding extensive mucociliary clearance [55]. Additionally, it has been shown in
318 several occasions [56,57] that the contact of nanoparticles with the surfactant present in the alveolar
319 zone leads to the coating of nanocarriers by a biomolecular corona, composed of lipids and proteins.
320 This corona affects nanoparticle hydrophobicity and possibly enhances biorecognition, with
321 consequences on the subsequent interactions with cells and other biological entities. Most works
322 report a negative impact of this process on the therapeutic outcomes. Interestingly, with regards to
323 the delivery of siRNA, recent works have suggested that modifying the surface of siRNA-loaded
324 nanoparticles with lung surfactant (by a simple incubation) provides improved siRNA transfer
325 activity due to facilitated cellular uptake [54]. Improved transfection efficiency of pDNA was also
326 reported previously in presence of lung surfactant [58]. siRNA-dendrimer (polyamidoamine,
327 generation 4) complexes of ~100-130 nm were microencapsulated in trehalose-inulin microparticles,

328 which displayed aerodynamic diameters of 4.5 – 5.5 μm , adequate to reach the deep lung. These
329 microparticles dissolved in aqueous medium, releasing the nanocomplexes, which showed enhanced
330 cellular uptake and transfection in RAW264.7 macrophages, comparing with native siRNA [59].
331 Protein-based molecules are, in many cases, regarded as sensitive and their manipulation in delivery
332 devices such as inhalers is often feared. A study demonstrated the stability of mRNA upon
333 nebulisation, showing no effect of nebulisation on protein duration of action or the cytotoxicity of the
334 formed PEI polyplexes [60].

335 Inhalable vaccines have also been the focus of several works and, although many pulmonary
336 vaccines have been proposed, only few involve nanocarriers. An interesting approach was reported
337 that uses a double emulsion formed by water/PLGA in organic/lactose-water, with IgG, the model
338 antibody, dissolved in the inner aqueous phase. The emulsion was spray-dried, resulting in PLGA
339 nanoparticles within lactose microparticles. Suitable properties for inhalation were observed, with
340 60% FPF. Submicron-particles were released after contact with aqueous medium, and approximately
341 70% IgG released after 6 days in pH 7.4 [53]. Another approach reported poly(glycerol adipate-co- ω -
342 pentadecalactone) (PGA-co-PDL) nanoparticles that were modified to express on their surface the
343 pneumococcal surface protein A, which is an important antigen of *S. Pneumoniae* (~20 mg antigen/mg
344 of nanoparticles). Nanoparticles of approximately 150 nm were then microencapsulated in leucine
345 microparticles to provide respirability. The latter registered MMAD of 1.7 μm and 74% FPF, which
346 grants the ability to reach the broncho-alveolar zone, potentiating the uptake by dendritic cells, as
347 demonstrated experimentally [61]. Silica nanoparticles were also reported for this end. Nanoparticles
348 were associated with plant-derived H1N1 influenza hemagglutinin antigen (HAC1) and proposed as
349 inhalable vaccine against influenza virus. A mucosal adjuvant (bis-(3',5')-cyclic dimeric guanosine
350 monophosphate (c-di-GMP)) was further tested. After IT vaccination of mice, the double-adjuvanted
351 vaccine (nanoparticles plus mucosal adjuvant) were observed to induce high systemic antibody
352 responses, comparable to the systemic vaccination control. Moreover, local IgG and IgA responses
353 were observed in the bronchoalveolar lavage [62].

354 The described works clearly demonstrate that the lung provides a suitable route for the delivery
355 of protein-based molecules, serving, in this context, the purpose of both systemic and local delivery.

356 2.2. Delivery of antibiotics

357 The delivery of antibiotics to the lung seems a very reasonable approach in the treatment of
358 infections that are based in that organ. In fact, the most common routes of delivery of antibiotics are
359 the oral and parenteral, even if the treatment of respiratory infections is intended. Addressing local
360 lung infections requires reaching effective concentrations of drug in the organ, which implies the
361 administration of significantly high doses and a general exposure of the organism to the drugs. The
362 direct administration to the infection site would, thus, permit using lower doses and avoid or
363 decrease systemic exposure, with the consequent reduction of systemic side-effects. Additionally, the
364 more targeted delivery is a premise to decrease the incidence of antimicrobial resistance, an important
365 current goal in antibiotic therapy [63,64]. Antibiotic resistance has been, for many years, one of the
366 greatest public health problems. The increasing misuse of these molecules, ever since their discovery,
367 has been making bacteria progressively resistant, by means of the development of specific cellular
368 mechanisms. This has been continuously and consistently posing a renewed challenge to the
369 treatment of infectious diseases [65].

370 The market makes available some formulations of inhaled antibiotics, including tobramycin,
371 colistin and aztreonam, which are mainly directed to the treatment of infections associated with cystic
372 fibrosis conditions [66]. Other applications have been reported occasionally, such as the use of
373 aerosolised antibiotics in hospital-acquired pneumonia [67]. Research in the area has been increasing
374 consistently and a recent review on inhalable antibiotic formulations is available in [66]. Along with
375 the discovery of new antibiotics, the development of delivery systems to improve the therapeutic
376 performance of the molecules has been object of scientific efforts and both approaches are, in fact,
377 effective countermeasures against antibiotic resistance. The search for new drug molecules is known
378 to be slower than the development of drug delivery systems that lead the antibiotics to the intended

379 site of action. Of the marketed formulations referred above, none is based on nanocarriers, but the
380 literature provides many works reporting their use to improve the performance of lung delivered
381 antibiotics, addressing, among others, the improvement of kinetic profiles and issues related with
382 side effects. One of the most popular respiratory infections is tuberculosis, caused by *Mycobacterium*
383 *tuberculosis*, which primarily accumulates and replicates inside alveolar macrophages located in the
384 alveolar zone of the lung [68]. Despite the existence of effective therapy of tuberculosis for many
385 decades, the fact is that it still remains a global epidemic, being a major healthcare problem, as
386 portrayed by the last data published by WHO [69]. Not only the established therapy is prolonged
387 and associated with severe side effects, which decreases therapeutic compliance, but also the issues
388 of co-morbidity with HIV and the existing bacterial resistance are relevant. A great number of works
389 propose the use of nanocarriers for tuberculosis treatment, in many cases envisaging lung delivery
390 applications. Very frequently, the developed carriers involve strategies of surface chemical
391 functionalisation, namely mannosylation. The rationale behind this approach is based on the fact that
392 bacterial hosts, the macrophages, have several surface receptors that are likely to be used as
393 therapeutic targets [70,71]. The mannose receptor is one of the main, which may provide a favourable
394 interaction with some units and chemical groups present on the carriers' surface, including the
395 mannose units, but also others like fucose and *N*-acetylglucosamine [72]. In principle, considering
396 that the bacteria are hosted by the macrophages located in the alveoli, this is the zone to be reached
397 in the design of any strategy aimed at treating tuberculosis by lung delivery.

398 SLN have been proposed as carriers for this end. Rifabutin-loaded SLN prepared with glyceryl
399 dibehenate (~100 nm) were further encapsulated in mannitol microparticles to acquire adequate
400 aerodynamic properties to reach the alveolar zone (~44% of particles with less than 6.4 μm). An *in*
401 *vivo* test in a murine model of infection (*Mycobacterium tuberculosis* strain H37Rv) demonstrated that
402 the inhalation of the dry powder permitted effective delivery of the antibiotic to the lung, along with
403 drug distribution to liver and spleen. Moreover, an enhancement of antibacterial activity was
404 observed compared to nontreated animals [43]. Another formulation of SLN, this time composed of
405 palmitic acid and cholesteryl myristate and loaded with rifampicin (~ 400 nm), was further freeze-
406 dried to obtain an inhalable powder. MMAD around 5 – 7 μm and FPF within 30% and 50% were
407 determined. The SLN were mannosylated to improve their targeting ability, which was verified
408 experimentally, with increased macrophage uptake (~80%) compared to non-functionalised SLN
409 (~40%) [71,73]. Rifampicin was also the chosen antitubercular drug to encapsulate in polymer-
410 glycosomes, which showed to be more stable than conventional liposomes [74]. These are
411 phospholipid/glycerol vesicles combined with trimethyl chitosan or hyaluronic acid (80 – 110 nm).
412 Upon nebulisation, MMAD of approximately 4 μm was obtained along with FPF up to 77%. In any
413 case, the aerodynamic performance of the carriers was always better than that of the free drug and
414 drug incorporation in the vesicles was found to increase its efficacy against *Staphylococcus aureus*.
415 Following IT administration to rats, glycosomes promoted the accumulation of rifampicin in the
416 lung, with lower systemic distribution, and low accumulation in other organs. The formulation
417 containing hyaluronic acid was found to perform more favourably [75]. Although it was not
418 discussed, the use of hyaluronic acid might be beneficial due to a favourable interaction of its *N*-
419 acetylglucosamine units with CD44 [76,77] and mannose receptors [78]. Chitosan and chitosan-folate
420 were further used to functionalise oleic acid-based nanoemulsions loaded with rifampicin, which
421 were nebulised to render adequate respirability (MMAD of 3 – 4 μm and FPF of 62-73%). It was found
422 that chitosan-folate provided increased cell internalisation, proposed to result from a favourable
423 interaction with macrophages by both chitosan units and folate groups. Additionally, this
424 formulation provided *in vivo* higher lung drug content and reduced plasma drug concentration [79].
425 Chitosan nanoparticles prepared by ionic gelation with tripolyphosphate were also proposed a
426 couple of times as carriers in antitubercular drug delivery. A first work described the association of
427 isoniazid and used spray-drying with lactose and leucine to reach an FPF of 45% [80]. More recently,
428 similar nanoparticles associated bedaquiline (size varying within 70 and 700 nm depending on
429 preparation conditions). A powder form of the nanoparticles was obtained by freeze-drying,
430 registering 28% FPF and 3.38 μm MMAD, which was better than the conventional DPI formulation

431 used as control (15% FPF and MMAD of 4 μm). The study determined absence of toxicity of the
432 nanoparticles *in vivo* in rats and further demonstrated higher drug concentration in lungs upon
433 inhalation of the microencapsulated nanocarriers [81]. Frequently, the choice of chitosan as
434 nanoparticle matrix material is not explicitly justified, leaving the readers with the sensation that the
435 polymer is only used because of its high popularity, a natural consequence of its favourable
436 properties regarding mucoadhesion and absence of toxicity. In this latter work, the authors justified
437 the positive results with a possible favoured uptake of nanoparticles by alveolar macrophages
438 mediated by an interaction of chitosan positive charges (from amino groups) with the negatively
439 charged surface of macrophages. However, most of the works fail to point out that the strong affinity
440 of macrophages by chitosan is possibly a result of the recognition of *N*-acetylglucosamine units of the
441 polymer by macrophage surface receptors, as was proposed in a work from our group reporting
442 chitosan microparticles as antitubercular drug carriers [78,82]. The use of chitosan as matrix material
443 was also proposed in genipin-crosslinked carboxymethylchitosan nanoparticles loaded with isoniazid
444 and rifampicin, which were freeze-dried to obtain a powder. After inhalation by rats, a greater
445 accumulation of drugs was observed in the lung upon delivery of the carriers compared with the free
446 drugs. Additionally, extended residence time of drugs in the lung was achieved and lower levels in
447 other organs (liver, kidney) were registered [83].

448 As a whole, several nanoparticle-based formulations are proposed in the frame of tuberculosis
449 therapy, in most cases showing improved results attributed to specific functionalisation of their
450 surface or benefits from their proper composition (e.g. chitosan). In order to provide adequate
451 respirability, the nanocarriers are either nebulised or transformed in inhalable powders using spray-
452 or freeze-drying. In the works showing *in vivo* results, the delivery by inhalation typically provided
453 increased lung concentrations of the drug and lower systemic exposure.

454 Other lung diseases work as a door for opportunistic infections, cystic fibrosis being a major
455 example. This is a genetic disorder caused by mutations in the cystic fibrosis transmembrane
456 conductance regulator (CFTR) gene. This gene is of the utmost importance, as it encodes a protein
457 that forms an ion channel in epithelial cell membranes. The genetic disfunction may translate into
458 different defects of the protein, in any case ending up in bronchial obstruction that occurs due to the
459 secretion and accumulation of a thick and sticky mucus in the airways. The accumulation of mucus
460 creates the adequate conditions for bacterial colonisation, which typically involves *Pseudomonas*
461 *aeruginosa* and *Staphylococcus aureus* [84-86]. This justifies that cystic fibrosis therapy requires regular
462 administration of antibiotics, apart from bronchodilators and mucolytics.

463 A solution of tobramycin for inhalation was the first approved aerosolised antibiotic to be used
464 against *P. aeruginosa* and, recently, a dry powder form of tobramycin has become available. However,
465 this drug shows poor mucus penetration, rapid clearance and suboptimal concentrations at the site
466 of infection, which are frequently not enough to stop the complications derived from the bacterial
467 infection [87]. The need for better therapies is one of the emergent objectives in the field of cystic
468 fibrosis. Nanotechnology can bring forth some solutions in this context. Mucus penetration is, indeed,
469 a major issue. If it is possible to overcome this barrier, enabling a more effective delivery of drugs,
470 infections can be eliminated with higher efficiency. In a very interesting work, Schneider et al. (2017)
471 demonstrated that mucus penetrating nanoparticles (polystyrene nanoparticles coated with
472 polyethylene glycol - PEG) of size up to 300 nm have higher retention in the lung and more uniform
473 distribution compared with similar sized nanoparticles devoid of PEG and, thus, mucoadhesive [88].
474 Regretably, no biological assays were reported so far, either *in vitro* or *in vivo*. Colistin was
475 encapsulated in PLGA nanoparticles which were further surface-modified with chitosan (270 nm) or
476 polyvinyl alcohol (PVA, 330 nm) and then spray-dried to reach adequate aerodynamic properties.
477 MMAD less than $< 5 \mu\text{m}$ was obtained when lactose was used as carrier, while the use of mannitol
478 resulted in MMAD $< 8 \mu\text{m}$. *In vitro* assays revealed increased ability of chitosan-modified particles to
479 penetrate artificial mucus and also suggested a role of the nanoparticles in potentiating the anti-
480 biofilm activity of colistin, possibly due to the ability of nanoparticles to penetrate the biofilm and to
481 sustain drug release [89]. A previous work from the same group, where tobramycin was encapsulated
482 in similar nanoparticles, demonstrated *in vivo* that PVA-modified nanoparticles reached the alveoli,

483 while particles modified with chitosan tend to appear in the upper airways, possibly as a consequence
484 of their specific aerodynamic characteristics [90]. Ciprofloxacin was self-assembled with PEG-g-
485 phthaloyl chitosan nanoparticles (218 nm) and further microencapsulated by spray-drying in
486 swellable alginate microparticles (volume mean diameter of 3.9 μm). Upon IT delivery to rats, the
487 encapsulated molecule was found in higher concentration in lung tissue and lung lavage comparing
488 with the administration of the control consisting of a physical mixture of lactose and micronised drug
489 [91].

490 Importantly, many bacteria regulate pathogenicity via a cell-to-cell communication system that
491 is known as quorum sensing. This is dependent on cell density and involves the production of
492 virulence factors to coordinate group behaviours [92]. Antibacterial strategies based on the inhibition
493 of quorum sensing are currently growing and this represents, indeed, a novel form of therapy. A very
494 interesting approach in the delivery of antibiotics for the treatment of *Pseudomonas aeruginosa*
495 infection involved SLN (<100 nm) loaded with a quorum sensing inhibitor. Nebulisation has resulted
496 in MMAD of 2.2 μm and FPF around 85% was determined, enabling the deposition of a certain
497 fraction in the bronchial region. The SLN demonstrated to penetrate into artificial sputum, but the
498 most important finding was that the proper SLN have anti-virulent effect, acting in addition to the
499 quorum sensing inhibitor to decrease the virulence factor pyocyanin [93].

500 As can be verified, under the scope of antibiotic delivery, a great deal of attention is given to
501 tuberculosis. Anyway, the number of works addressing antibiotic delivery mediated by nanocarriers
502 that present a certain degree of multifunctionality while providing real demonstration of potential
503 for lung delivery, represents only a fraction. Apart from tuberculosis, *Pseudomonas aeruginosa* and
504 *Staphylococcus aureus* are the two main targets, being frequently associated with cystic fibrosis and
505 pneumonia, although they can be also involved in hospital-acquired lung infections, for instance. It
506 was demonstrated in the several works described that the nanocarriers can provide extra strength to
507 antibiotic-mediated therapies.

508 2.3. Applications in cancer therapy

509 The WHO refers to lung cancer as one of the most lethal [94]. In 2018, 18.4% of cancer-related
510 deaths were of lung cancer, and the number of new cases (11.8%) was one of the highest, on par with
511 breast cancer [95]. WHO has a set of goals to fight cancer aggressively, and the development of new
512 strategies in cancer treatment is a priority worldwide. Lung cancer can be categorised into non-small
513 cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is considered aggressive and
514 comprises approximately 85% of all occurrences, in which various subtypes are included such as
515 adenocarcinoma and squamous cell lung cancer. SCLC is even more aggressive, comprising the
516 remaining 15% of cases [96]. The high probability of metastasis derived from SCLC, and the
517 frequently late diagnosis, contribute to the high mortality [97,98]. At earlier stages, the treatment for
518 both types of lung cancer is surgery, enabling the removal of the affected area. However, at later
519 stages, chemotherapy and radiation are the valid options, often to reduce the tumor mass before any
520 surgical procedure [97,99]. Regretably, these options have great impact on patient's physiology, as
521 both cancerous and healthy cells are attacked, resulting in symptoms that are difficult to manage. As
522 a consequence, patient susceptibility to other diseases is increased.

523 The scientific community has been working to develop more targeted therapies, which is
524 facilitated by the increasing information on molecular pathways, specific receptors and cancer
525 microenvironment, enabling different treatment approaches. Although the intravenous route is the
526 most used to deliver anticancer drugs, inclusive in lung cancer, the use of the lung route is an
527 alternative yet to be fully explored in lung cancer therapy. This approach would allow a more
528 targeted delivery, reaching directly the affected area, possibly with higher effectiveness than that
529 provided by systemic delivery. Importantly, the lung can be considered the main route for the
530 delivery of anticancer drugs in cases of lung cancer, but can also be used as add-on therapy for the
531 treatment of lung metastasis secondary to other cancers. Overall, it is considered that this approach
532 would potentially enable the use of lower doses of anticancer drugs, with reduced systemic exposure

533 and consequent residual metabolisation of the molecules [100]. This strategy further helps on the
534 reduction of adverse effects, contributing for the increased quality of life of the patients.

535 The number of nanocarriers proposed for an application in lung cancer mediated by lung
536 delivery is high. In most cases, a therapeutic effect is envisaged, but some of the works address
537 diagnostic purposes. Although this is of great importance in cancer, especially at early stages of
538 development, these strategies will not be detailed further, as they are out of the scope of the review.
539 For further reading on this matter, Silva et al. (2019) and Mottaghitalab et al. (2019) comprise two
540 comprehensive reviews on potential diagnostic strategies [101,102]. Therefore, only works on
541 nanocarriers envisaging therapeutic approaches will be considered.

542 The general observation of the literature indicates that, in most cases, the proposal of
543 nanocarriers for an application in cancer therapy implies functionalisation, that is, carriers with some
544 sort of surface modification that benefits their interaction with the tumour environment. One of the
545 strategies often reported in this context relies on the use of a matrix that is added of molecules
546 potentially recognised by cell receptors prevailing in cancer cells comparing with healthy cells. Such
547 an approach was already discussed briefly in the previous section, referring to carriers endowed with
548 cell targeting ability mediated by mannose moieties. In the context of lung cancer, lactoferrin-
549 chondroitin sulfate nanocomplexes (~190 nm) were reported to co-deliver doxorubicin (Dox) and
550 elagic acid. The latter was first converted into water soluble nanocrystals due to its hydrophobicity.
551 The nanocomplexes were prepared by electrostatic interaction between lactoferrin and chondroitin
552 sulfate and the two drugs incorporated during this process. Due to the overexpression of CD44 and
553 lactoferrin receptors on the surface of lung cancer cells, these nanocomplexes were shown to have
554 favoured cell recognition, mediated by chondroitin sulfate and lactoferrin content, respectively. The
555 authors further hypothesised that clathrin-mediated endocytosis could have contributed favourably
556 to the internalisation of nanocomplexes, as their size is within the range of the pore size of the clathrin
557 receptor (up to 200 nm) [103]. Therefore, the functionality of these carriers is provided not only by
558 their size but also by their composition, which ensures specific targeting ability. To provide adequate
559 aerodynamics for lung delivery, the nanocomplexes were then microencapsulated into a mannitol
560 matrix, reaching FPF close to 90% and MMAD of 2.56 μm . After IT insufflation of the
561 microencapsulated nanocomplexes in tumour-bearing mice, tumour growth biomarkers were
562 quantified and revealed lower levels when the inhalable formulation was used, in comparison with
563 the inhalation of free drugs or intravenous administration [42].

564 These cell recognition strategies were also addressed in works with gold nanoparticles. Such
565 carriers have strong interest in cancer therapy, finding applications in photothermal therapy,
566 radiotherapy and also as drug carriers. Their inhalation has been demonstrated to provide lung
567 accumulation, which can be useful in lung cancer therapy [104]. A very recent review on the topic is
568 available in [105]. Gold nanoparticles (2 nm) that were coated with functional derivatives of thiolated
569 PEG have shown invisibility towards the immune system provided by PEG [106], but also enabled
570 attaching other moieties to provide specific targeting. The surface of the nanocarriers was thus
571 modified with the ligand RGD, a peptide with relatively high and specific affinity for integrins
572 overexpressed in tumour neovasculature [107,108]. A mice model of single-nodule lung
573 adenocarcinoma [109] was used to establish which route of administration, either inhalation or
574 intravenous delivery, would be more effective on adenocarcinoma targeting using the nanocarriers.
575 The biodistribution data demonstrated higher concentration of the carriers upon inhalation [110]. In
576 another approach, gold nanoparticles were loaded with temozolomide (~40 nm), an alkylating agent
577 already in use in other cancer types. The IT administration to healthy mice indicated the safety of
578 gold nanoparticles upon quantification of lactate dehydrogenase and the tumour markers
579 carcinoembryonic antigen and alpha-fetoprotein. The proper carriers were reported to induce
580 oxidative damage and ability to inhibit cell proliferation and cell cycle in G1-phase, while the delivery
581 of drug-loaded carriers to mice bearing lung cancer demonstrated a synergic effect between the
582 carriers and the loaded drug [111].

583 The optimisation of the interaction of nanocarriers with cancer cells has also been reported using
584 SLN. A complex nanodelivery system based on SLN was proposed, being composed of multi-

585 compartmental lipid nanocomposites (190-225 nm). Berberin and rapamycin, with demonstrated
586 synergic anticancer effect, were initially encapsulated in SLN. To optimise the rate of delivery of both
587 drugs, multicompartiment systems were developed. Berberin was incorporated as hydrophobic ion
588 pair with sodium dodecyl sulfate in SLN's core, sustaining its release, while rapamycin was pre-
589 formulated as phospholipid complex, thus helping to improve its solubility and relatively enhance
590 its release. The tumour targeting ability was improved by layer-by-layer assembly of the cationic
591 lactoferrin and the anionic hyaluronic acid, which target the CD44 and lactoferrin receptors
592 overexpressed by lung cancer cells. Adequate aerodynamics were achieved after spray-drying with
593 a mixture of mannitol/maltodextrin/leucine (MMAD of 3.3 μm , FPF of 56%). An assay in mice bearing
594 lung tumour demonstrated that inhaled nanocomposites induced a decrease of lung weight
595 comparing with the inhalation of free drugs, along with reduction of tumour size and levels of
596 angiogenic markers [112]. Another work proposed the modification of SLN surface with a chitosan
597 derivative that was previously added of folate moieties [113]. The authors hypothesised that both the
598 chitosan derivative and the folate engraftments would increase the retention of the nanoparticles
599 within the lungs, and activate the folate receptors, increasing the amount of drug delivered to cancer
600 cells. The nanocarriers (~250 nm; +32 mV) provided slower release of paclitaxel after coating (58% in
601 3 days) and demonstrated binding affinity to cell lines expressing the folate receptor. In *in vivo* assays,
602 higher lung paclitaxel concentration was observed for the inhaled chitosan-coated SLN compared
603 with the intravenous administration of the drug. Moreover, drug concentration was higher at 1 h and
604 6 h post-administration for the coated formulation compared with inhaled and intravenous delivered
605 paclitaxel. As a final remark for this study, the authors noticed that the SLN were distributed
606 throughout the solid lung tumours, with low interaction with the vessels, which occurs with systemic
607 delivery of anticancer agents. Paclitaxel was also loaded in PEG-poly(lactic acid) (PLA) nanoparticles
608 that were further conjugated with the epithelial cell adhesion molecule (EPCAM, CD326), also
609 overexpressed in lung cancer. IT delivery of nanoparticles to c-Raf transgenic lung cancer mice
610 permitted reducing drug toxicity, with animal surviving increasing from 20% to 70% [114]. Another
611 approach proposed lipid polymeric nanoparticles (hydrophobic polymeric core, phospholipid layer
612 and an outer layer of epidermal growth factor (EGF), PEG and distearoylphosphoethanolamine)
613 targeting the EGF receptor (EGFR) [115], which is overexpressed in lung carcinoma [116,117].
614 Cisplatin and Dox were the associated drugs. The presence of EGF in the outer part of the
615 nanoparticle promoted the interaction with EGFR, leading to the release of drugs at the cancer site.
616 An *in vivo* assay revealed tumour inhibition ratio of ~75%.

617 Chitosan-coated PLGA nanoplexes were proposed to carry an antisense oligonucleotide against
618 the human telomerase RNA component, as telomerase activity is detected in most NSCLC. The
619 potential of the oligonucleotide as a telomerase inhibitor has been described [118], although its poor
620 cellular uptake hinders its use in cancer therapy. The nanoplexes (± 160 nm) were delivered IT to
621 healthy mice, using the model of the isolated perfused and ventilated lung, and provided increased
622 uptake of the oligonucleotide by the epithelium than that observed for the free form of the
623 oligonucleotide. Although no specific study was performed, the authors justified the results with the
624 potential ability of nanoparticles to escape pulmonary clearance mechanisms [119].

625 A solution towards a resistant form of cancer was proposed as inhalable self-assembled
626 nanoparticles comprised of human serum albumin (HSA), tumor necrosis factor (TNF)-related
627 apoptosis-inducing ligand (TRAIL) and Dox [120]. The latter was conjugated to HSA and formed
628 nanoparticles, which were then coated with TRAIL (342 nm). Initial tests in H226 cells, which are
629 representative of NSCLC, have shown that the simultaneous presence of Dox and TRAIL enabled
630 increased cytotoxic potential, as cell viability after 3 days of exposure decreased from approximately
631 60% when only one of the molecules was present in HSA nanoparticles, to 20-30% after dual
632 association. An *in vivo* assay was then performed in lung tumour-bearing mice, delivering the
633 nanoparticles in the form of micron-sized liquid droplets. The tumours of mice treated with HSA
634 nanoparticles combining TRAIL and Dox were much smaller and lighter than those of mice treated
635 with the corresponding nanoparticles containing only one of the molecules, TRAIL or Dox.
636 Haloperidol was also used as ligand to enhance targeting ability of albumin-based nanoparticles (218

637 nm). Nanoparticles were prepared by desolvation of bovine serum albumin, previously conjugated
638 with haloperidol and loaded with Dox. Spray-drying with mannitol, trehalose and leucine resulted
639 in nano-in-microparticles with aerodynamic diameter of 4.6 μm and FPF of 66% [121].

640 Some works further report therapeutic approaches that rely on the ability of the carrier matrix
641 to respond to different stimuli [122,123]. Modification of temperature and pH are usual stimuli to be
642 used [124], setting the basis for the elaboration of the so called *smart polymers* or *systems*. The rationale
643 behind their use is that a certain stimulus (pH value or temperature that is reached), will trigger a
644 phase transition in the carrier matrix, leading to the release of the drug in a predetermined site. In
645 this context, a copolymer based on methoxy poly(ethylene glycol)-poly(ethylenimine)-poly(L-
646 glutamate) was produced and nanoparticles prepared (< 75 nm), using electrostatic interaction and
647 chelate effect to encapsulate simultaneously Dox and cisplatin [125]. *In vitro* assays demonstrated
648 increased release of Dox at acidic pH, showing the capacity to release the drug in a cancer setting.
649 The pulmonary administration of the nanocarriers to mice with metastatic lung cancer was
650 performed using a Microsprayer aeroliser, resulting in increased accumulation of carriers within the
651 lungs, along with low concentration in other tissues, especially in the area surrounding tumour
652 lesions. It was hypothesised that the smaller size of the carriers benefitted their penetration in the
653 cancer mass, while an ineffective vessel arrangement prevented a systemic dissemination. The
654 results also shown decreased tumour masses, suggesting increased efficacy of the nanoformulation.
655 Dendrimers of poly(amidoamine) were also used in a similar pH stimulation strategy. Dox was
656 conjugated with the polymer and the dendrimers were spray-dried with mannitol to endow suitable
657 aerodynamic characteristics (FPF was 40-60%). Dendrimers readily released from microparticles in
658 aqueous medium and drug release was only found to occur in response to intracellular pH drop [126].
659 In another work, similar dendrimers showed strong time-dependent toxicity in Calu-3 cells, a model
660 of the respiratory epithelium, which was attributed to sustained drug release. It was also shown that
661 the conjugation of PEG molecules to the dendrimer improved their permeation across the cell layer,
662 in a concentration-dependent manner. In this case, the dendrimers were formulated in a pressurised
663 metered dose inhaler, leading to aerosols with 82% FPF and MMAD of 1.3 μm [127]. Dendrimers of
664 PEGylated polylysine, also conjugated with Dox, were IT administered to a syngenic rat model of
665 lung metastasised breast cancer. An administration twice a week led to over 95% reduction in lung
666 tumour after two weeks, comparing with IV administration of Dox solution, which resulted in a
667 reduction of 30%-50% [128].

668 In some cases, a combination of the above-mentioned strategies is proposed, as happens in a
669 work reporting a stimuli-responsive core-shell nanoparticle conjugated with folic acid. The rationale
670 behind this formulation was to create a pH- and temperature-sensitive network, comprised by a
671 copolymer of poly(*N*-isopropylacrylamide) and carboxymethylchitosan, which comprises the shell
672 of the nanosystem. In turn, the core is comprised of PLGA and an image contrast agent
673 (superparamagnetic iron oxide, SPIO). While PLGA allows the controlled release of the encapsulated
674 molecules, gemcitabine in this case, SPIO serves the dual role of contrasting agent and inductor of
675 temperature change by external application of an alternating magnetic field. SPIO-induced
676 temperature alterations lead to conformational change of polymeric shell, allowing drug release.
677 Additionally, the shell of the system is pH-sensitive, providing drug release at the acidic pH
678 characteristic of cancer environment. Moreover, the delivery is even more targeted owing to the
679 surface conjugation with folic acid, benefitting from the overexpression of the folate receptor in
680 cancer cells [129]. The nanocarriers (~289 nm, -36 mV) evidenced increased cell uptake in the presence
681 of a magnet, as a consequence of the presence of SPIO in the formulation. *In vivo* assays in lung
682 tumour-bearing mice show decreased tumour volume comparing with the controls. Pulmonary
683 retention of nanoparticles was confirmed by magnetic resonance imaging (MRI) and, when coupled
684 with radiotherapy, a synergic effect takes place to slow tumour growth.

685 Finally, magnetic nanoparticles have also been proposed several times within the scope of lung
686 cancer. Many reports explore an application in lung cancer diagnosis, using Fe_3O_4 paramagnetic cores
687 [130] or gadolinium-based particles [131,132], in the latter further enabling a radiosensitising effect.
688 Nevertheless, therapeutic actions are also proposed. Iron oxide nanoparticles (Fe_3O_4 ; 56 nm, -49 mV)

689 were spray-dried with lactose and doxorubicin, reaching an MMAD of 3.27 μm . An *in vitro* study
690 demonstrated that, comparing with a liquid suspension, the microencapsulated nanoparticles
691 provided more than twice deposition and retention of particles in regions under the influence of a
692 strong magnetic gradient [133].
693

694 As a whole, several different strategies are described that end up with positive results in lung
695 cancer treatment. Nevertheless, cancer research still has much ground to cover and the associated
696 therapeutics are growing at the rate of decipheration of new receptors and new molecular cascades.
697 Nanotechnology is progressing along these discoveries, to provide improved strategies of cancer
698 treatment. Those described above are dominated by the optimisation of the carriers surface, either by
699 engineering with specific ligands, by carefully selecting the matrix components or by combining all
700 the effects, in order to provide more targeted delivery of the drugs and an intimate contact with
701 cancer cells, which will thus result in improved therapeutics.
702

703 3. Expectations for the future

704 Pharmaceutical technology has been playing a vital role on medicine, as it allows exploring
705 different materials and their combinations to prepare drug carriers and to further endow these with
706 better properties that enable reaching the desired target sites, ending up with therapeutic success.
707 Many questions arise around the topic of nanocarrier-based lung drug delivery. Scientists have been
708 giving many and varied answers, in the attempt to address all the rising issues, finding alternatives
709 and engineering adequate systems to fulfill requirements and needs. One of the concerns is always
710 the fate of the drug. In lung drug delivery, the objective is sometimes to retain the drug in the lung,
711 as happens in local delivery approaches, thus minimising the systemic absorption. In other cases, a
712 systemic effect is the desired outcome and the carriers are engineered to avoid retention. The options
713 to address therapeutic demands are varied, as seen by the plethora of systems, alternatives and
714 engineering possibilities described through the review. As expected, the global analysis reveals that
715 most of the works focus on the use of the lung route to attain local rather than systemic effects. It was
716 also verified that, from the three main topics explored in the review, the delivery of anticancer drugs
717 is the one concentrating more proposals in the literature, which is justified by the severity of the
718 numbers associated with this disease, the increasing number of patients and the lack of therapeutic
719 options, apart from the marketing appeal of cancer therapies.

720 Whichever the specific topic, it has become clear that the use of nanocarriers is an added value
721 and may provide an evolution of therapeutic responses if used properly and the arising toxicological
722 concerns are addressed. Furthermore, the engineering of different strategies mainly involved surface
723 functionalisation of the carriers or at least took benefit of their components to provide specific effects.
724 The latter is perhaps the strategy still requiring further investment to bring out all its potential, as
725 *smart polymers* have emerged as a new range of powerful tools, but still need refinement. The use of
726 the different physiological conditions, such as pH, temperature and redox compounds or even light
727 at different wavelengths can be the answer to more targeted and efficient therapies. Objectively, the
728 field needs clinically feasible formulations, which possibly could combine some of the different
729 strategies that were described, certainly using some kind of surface engineering to reach specific
730 biological targets, but also adjusting the desired properties with the use of materials that may respond
731 specifically, either to stimuli or to the established characteristics of the target area. Recently, a very
732 interesting and promising approach was reported in this context, comprising a nanoparticle-in-
733 microgel system that provides drug release triggered by the presence of proteases. As the presence
734 of these enzymes is greatly increased in the lung as a consequence of the inflammatory process
735 related to asthma, COPD and cystic fibrosis, this may comprise a therapeutic strategy for the
736 treatment of these conditions [134]. Despite the tremendous advancement, the evolution of the field
737 is in strong dependence of new knowledge being generated at more basic science, namely the
738 molecular mechanisms of the diseases, which are great indicators of tools to be used in the
739 development of new therapies. Additionally, it cannot be forgotten that many of the tools identified

740 as successful and providing improved therapeutic responses, that is, carriers and above all, the
741 materials, are not approved so far by the regulatory entities for an application in lung delivery. This
742 poses a great challenge itself. Addressing the toxicity of inhaled therapeutic nanocarriers is actually
743 a matter of inescapable importance. For many years now it has become clear that the biocompatibility
744 of nanomaterials is not that of the raw materials and its evaluation needs to go much beyond the
745 assessment of the isolated components. The nanomaterial must be considered a new entity instead,
746 within the context of a specific delivery route [135]. Therefore, generating data on the safety of the
747 nanocarriers and the new materials identified as potential adjuvants, in the framework of the lung
748 route, is currently understood as an urgent need to potentiate lung drug delivery applications. This
749 should involve toxicity tests that evaluate all the possible toxicity pathways, both *in vitro* and *in vivo*,
750 while ensuring that the 3Rs policy to reduce, refine and replace the use of animals in research is
751 followed. The initial *in vitro* tests should address cytotoxicity and genotoxicity, and should also
752 evaluate potential epigenetic toxicity [136]. The fate of the proper carriers after the delivery is often
753 disregarded. A very recent study comparing the clearance kinetics of liposomes and solid lipid
754 nanoparticles after IT delivery of suspensions to rats has shown similar clearance rates, despite
755 different deposition patterns [137]. Studies around this topic are, thus, imperative to provide data on
756 the safety of the materials and the kick-off to their clinical application.

757 It is important to point out that, when *in vivo* assays were described, which occurred in a
758 considerable number of the presented works, IT delivery of the nanocarriers was the predominant
759 technique for the assessment, which implies a high risk when establishing possible correlations with
760 human delivery. The fact that nanocarriers themselves do not exhibit suitable aerodynamics for
761 inhalation is also a matter of relevance, as this always implies an extra step, typically proposed to
762 involve the spray-drying of nanocarriers to produce nano-in-microcarriers that can deposit in the
763 lung.

764 The area still needs to evolve in several topics before inhalable nanocarriers enter clinical trials.
765 Not only the question of performing more realistic *in vivo* assays is determinant, but also the
766 toxicological assessment plays a defining role. Helpful technologies have been arising, such as 3D
767 printing, which was used on the printing of artificial airways that enable the study of particle
768 flowability and dose assessment. Lim *et al.* describe this application in the neonate, showing a
769 powerful tool to improve the ethics associated with formulation testing and to provide solutions to
770 children born with respiratory complications [138].

771 All in all, this review highlighted an integrative process that considers progress made at the level
772 of basic science, which clarifies pathophysiological aspects of each clinical condition, and the
773 development of tools and strategies to reach the pharmacological targets. Many works were
774 described with inhalable nanocarriers that have been showing potential, even with the existing
775 limitations. All the issues, however, point out to a common objective of providing the knowledge to
776 enable the engineering of nanocarriers that will promote improved lung therapeutics.

777

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784 References



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