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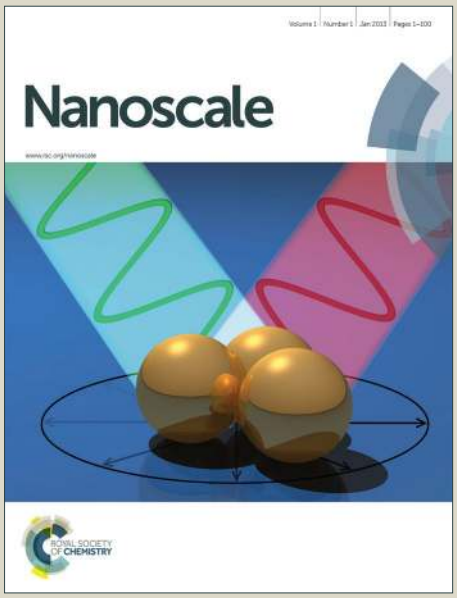

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Recent Advances in Chitosan-Based Nanoparticulate Pulmonary Drug Delivery

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Abstract

The advent of biodegradable polymer-encapsulated drug nanoparticles has made the pulmonary route of administration an exciting area of drug delivery research. Chitosan, a natural biodegradable and biocompatible polysaccharide has received enormous attention as a carrier for drug delivery. Recently, nanoparticles of chitosan (CS) and its synthetic derivatives have been investigated for the encapsulation and delivery of many drugs with improved targeting and controlled release. Herein, recent advances in the preparation and use of micro-/nanoparticles of chitosan and its derivatives for pulmonary delivery of various therapeutic agents (drugs, genes, vaccines) are reviewed. Although chitosan has wide applications in terms of formulations and routes of drug delivery, this review is focused on pulmonary delivery of drug-encapsulated nanoparticles of chitosan and its derivatives. In addition, the controversial toxicological effects of chitosan nanoparticles for lung delivery will also be discussed.

Key words: chitosan, chitosan derivatives, nanoparticles, inhalation, biodegradable, pulmonary drug delivery

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1. Introduction

Drug delivery directly into the lungs is an efficient method to achieve both local and systemic effects for therapeutic agents. The advantages of pulmonary drug delivery include superior efficacy, low toxicity, and sustained and rapid onset of action, which other drug administration routes (except injections) cannot achieve. The large epithelial surface area, the high organ vascularization, extremely thin absorptive alveolar epithelial membrane, the immense capacity for solute exchange, and good blood supply are notable factors that make the lung an ideal drug delivery route for the treatment of both local and systemic disorders. Delivery of drugs into the lungs ensures low systemic side-effects compared with oral or intravenous administration and no first-pass metabolism, which is a common problem for orally administered drugs. Direct delivery of drugs into the lungs also enables lower doses with an equivalent therapeutic action compared to oral or parenteral routes.[1] For efficient pulmonary delivery, aerosol particles containing drug should be small enough (<5 □m) to avoid contact with the upper respiratory tract and reach the alveolar space within the deep lung. Although not fully established, further reduction in size to the nm range results in increased deep lung deposition, with nanoparticles of the size <100 nm found to be particularly efficient.[2]

Many biodegradable polymeric micro/nanocarriers have been extensively investigated for lung delivery of various drugs.[3, 4] For example, poly(lactic-co-glycolic) acid (PLGA),[5-8] poly(ethyleneglycol) (PEG)[9], polyethylenimine (PEI)[10] and chitosan (CS) [8, 11-13] have been studied for pulmonary drug delivery. The hydrophobic character of PLGA limits the encapsulation efficiency and subsequent delivery of hydrophilic drugs in PLGA based nanoparticles.[14] The hydrophobicity can be reduced by attaching hydrophilic PEG groups onto the PLGA backbone and such approaches have opened a window for developing various

types of PEG-PLGA copolymers for encapsulating both hydrophilic and hydrophobic drugs. Depending on the molecular weight, PEG alone can encapsulate mostly hydrophilic drugs and is capable of releasing the encapsulated drug into the target area. [15] On the other hand, CS can accommodate both hydrophilic and hydrophobic drugs due to its amphiphilic properties. [16] Among the natural polymers investigated for lung delivery of various drugs,[4] CS is recognised as a non-toxic, biocompatible (especially with respiratory cells)[17, 18] and biodegradable [17, 19, 20] polymer. The advantages of CS over other biodegradable polymers are that it is mucoadhesive [21-24] and an absorption enhancer.[24] CS has the ability to modify the morphology of particles and thus increase the dispersibility of particles leading to increased deposition into the lungs.[17] CS and its derivative *N*-trimethyl chitosan (compound **2, Figure 1**) are well known as permeation enhancers[25, 26] of various drugs. In addition, CS has inherent immunogenicity which is absent in other polymers, and this enables its use as an adjuvant for vaccine delivery into the lungs.[27, 28] Therefore, in this review, we have focused on the use of CS and its derivatives in investigating pulmonary delivery of various therapeutic agents, including small molecule drugs, proteins/peptides, vaccines and genes. The ongoing toxicological debate associated with CS nanoparticles (NPs) for lung delivery is discussed.

2. Chitosan, a promising polymer

Chitosan (CS, **1**) is a linear polysaccharide composed primarily of β-(1→4)-linked glucosamine (2-amino-2-deoxy-β-D-glucopyranose). It is obtained by deacetylation of naturally occurring chitin under basic conditions and thus contains varying amounts of *N*-acetylglucosamine (2-acetamido-2-deoxy-β-D-glucopyranose) due to incomplete deacetylation (Fig. 1).

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CS is insoluble in aqueous solutions at neutral pH; however, it is soluble in aqueous acidic media (pH<5) as the basic amino groups ($-NH_2$) are protonated ($-NH_3^+$) and therefore, it is positively charged. The cationic charge of CS provides mucoadhesive properties[13, 22, 23] that enable it to adhere to the mucosa of lung epithelial cells and contribute to the prolonged release of encapsulated drugs.[29-31] The positively charged CS has been found to improve drug absorption by opening the intercellular tight junctions of the lung epithelium.[24, 32] The mucoadhesive properties of spray dried CS microspheres containing montelukast sodium was found to increase with increasing concentrations of CS in the formulation.[33] It has also been reported to promote drug transepithelial transport in the alveolar area.[34] Furthermore, CS itself has been reported to have bactericidal properties.[35, 36]The antibacterial effects of CS have been attributed to an electrostatic interaction between the protonated amino groups of CS and the phosphoryl groups and lipopolysaccharides of bacterial cell membranes, which destroys the membrane and releases the cellular contents. These properties additionally aid the lung delivery of antibiotics containing CS against infectious diseases. Depending on the molecular weight, degree of deacetylation (DD) and hydrolysis time, CS undergoes enzymatic depolymerisation in the presence of lysozyme,[37] which is the most important antimicrobial enzyme present in secretions of the respiratory tract and that's why it has been studied extensively for delivering various antibacterial agents into the lungs, as discussed in section 4.1.

A comparative study on the oral versus pulmonary delivery of calcitonin in the presence or absence of different CS oligomers was carried out in a rat model. The hypoglycaemic effects of CS oligomers on drug absorption from pulmonary delivery were found to be stronger than that from the oral route.[38] This finding suggests the superiority of pulmonary calcitonin delivery over the oral route. CS has also been reported to directly activate the immune system

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due to its cationic nature[28] when used as a vaccine adjuvant,[39] suggesting potential applications in vaccine delivery. Studies on the lung delivery of CS based vaccines are described in section 4.5 of this review. Considering the above discussed background, it is clear that CS has profound applications as a promising pharmaceutical excipient[22] in drug delivery.

3. Toxicity of CS

To date, the safety issues of CS and its derivatives in pulmonary drug delivery systems have not been fully understood. Currently, there are no published human toxicity data on CS NPs. Therefore, the potential adverse effects of CS NPs in humans remain unclear. Various human airway cell culture models, e.g., bronchial Calu-3 cells and alveolar A549 cells, have been used to investigate the safety and efficacy of inhaled therapeutics with CS-based carriers; however, contradictory outcomes have been reported.[1, 17, 40-42] Recently, the toxicity of CS and leucine-conjugated CS (**5**, Fig. 2) NPs on BEAS 2B cells were studied. The conjugate and its NPs showed relatively more toxic and inflammatory effects than those of CS and its NPs. However, the level of toxicity was low (IC_{50} = 2 mg/mL following 48 h exposure) and thus **5** has potential utility for pulmonary drug delivery applications.[43]

The kinetics and toxicity of hydrophobically modified (with 5β-cholanic acid) glycol chitosan (**6**) NPs were investigated after intratracheal instillation of NPs to mice.[44] The NPs were rapidly taken up into the systemic circulation and excreted via the urine 6 h after intratracheal instillation. The NPs distributed to several extrapulmonary organs at very low levels and induced transient neutrophilic pulmonary inflammation from 6 h to day 3 after instillation. Following intratracheal nebulization in mice, NPs of PLGA coated with CS showed better biodistribution and low toxicity compared with non-coated NPs.[45] CS coated

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PLGA did not induce a significant inflammatory response as evidenced by low levels of cytokine release. The particle surface charge due to CS influenced the transporting of NPs across the mucus layer for better biodistribution. In another study, human alveolar epithelial cells incubated with CS-coated PLGA NPs containing DNA showed more than 90% viability at all concentrations tested.[46] CS based PLGA NPs also showed more than 80% cytocompatibility with alveolar epithelial cells up to a concentration of 1 mg/mL, which is similar to the outcome observed by Grenha et al.,[17] which produced up to 80% viability with Calu-3 cells and A549 alveolar epithelial cells up to NP concentrations of 1 mg/mL. Thus, the above studies suggest CS-coated PLGA NPs are safe for lung delivery of drugs. Overall, these findings support the use of CS based NPs as pulmonary drug delivery vehicles due to their excellent biocompatibility, low pulmonary toxicity, and rapid elimination.

It is difficult to simulate in vitro interactions of inhaled medicines with lungs cells. Therefore, whole animal models are required to assess the real toxicological effects of NPs. The toxicity of CS was found to be dependent on molecular weight, DD, and concentration. At high DD the toxicity is related to the molecular weight and the concentration; however, it is biologically less effective and less related to the molecular weight at lower DD. Thus, it is not straightforward to draw conclusions in terms of safety and toxicity issues due to the different DD, molecular weight, solubility and different assay conditions employed across different studies. Therefore, vigorous studies are warranted to obtain a clearer picture about the safe application of CS and its derivatives for pulmonary drug delivery.

4. Chitosan based lung drug delivery

CS based micro- and nanoparticles for pulmonary drug delivery have improved the therapeutic effects of drugs by modifying drug bioavailability. Drug encapsulation in micro-

and nanoparticles leads to reduced drug toxicity[1, 43] and prolonged biological half-life of drug. CS NPs prepared by cross-linking with tripolyphosphate (TPP);[47-51] or glutaraldehyde[52-54] (**7**, Fig. 2) have been demonstrated to enhance mucoadhesiveness or cellular absorption upon lung delivery, as discussed in the introduction section. Sustained delivery of CS encapsulated insulin NPs [49] and terbutaline sulfate [55] from DPI formulations have also been studied. CS based DPI formulations of beclomethasone dipropionate,[56] clindamycin[57] and salbutamol sulfate[57] showed promising controlled release profiles. A DPI formulation containing hydrophilic (terbutaline sulfate) and hydrophobic (beclomethasone dipropionate) drugs, CS (as a drug release modifier) and leucine (aerosolization enhancer) showed promising sustained release delivery of both drugs from a single formulation.[58] Lung delivery of microencapsulated protein,[48, 49] tobramycin,[7] and methotrexate[59] loaded CS NPs from DPI formulations have been investigated. CS has also been found to increase the stability of drug formulations for lung delivery from DPI.[12, 60-64]

The above studies demonstrate the potential of CS for lung delivery of drugs. Based on the published literatures, the types of drugs investigated for lung delivery with CS or CS-derivatives can be classified as antibacterial and antitubercular drugs, anti-asthmatic, proteins/peptides, anticancer drugs, genes (includes RNA-based therapeutics), vaccines and other drugs (see Figure 3), which further supports the wide applicability of CS in lung drug delivery. The motivations for investigating antibacterial/antitubercular drugs for lung delivery could be due to the bactericidal effect of the CS itself, which is an additional advantage for lung delivery of CS based drug formulations. A comprehensive list of CS based lung drug delivery systems is presented in Table 1. The well-established fabrication methods for preparing various drug encapsulated CS nanoparticles (ionic cross-linking, coacervation/precipitation, emulsion/double emulsion cross-linking, and self-assembly) will

not be discussed in detail herein as they have been reviewed previously.[65-67] The following sub-sections are dedicated to the pulmonary delivery of various categories of drug formulations investigated so far.

4.1. Antibacterial/antitubercular drugs

Antibacterial/antitubercular drugs are the largest class of drugs investigated for CS based pulmonary delivery (40%, Figure 3), partly because CS has bactericidal properties in its own right. Pulmonary delivery of levofloxacin against *Pseudomonas aeruginosa* using CS modified by grafting octanoyl chains onto the CS backbone (**7**) has been studied.[68] The highly dispersible dry powders of drug with varying concentration of CS and octanoyl CS **8** were prepared to study the effect of hydrophobic modification on aerosolization of the drug. Microparticles of **8** displayed mucoadhesive properties leading to increased drug residence time in the pulmonary mucus. An MTT assay performed on an A549 cell line indicated that the polymers and the formulations were non-cytotoxic. However, **8** showed a significantly lower minimum inhibitory concentration (MIC), 4-fold less than that of the commercially available CS, against *P. aeruginosa*. The reasons behind the lower MIC of **8** are unclear. In another study, spray dried levofloxacin loaded, glutaraldehyde-crosslinked CS (**7**) microspheres showed higher dispersibility and better antimicrobial properties against *P. aeruginosa* infections in cystic fibrosis compared with levofloxacilin alone[53] and the dry powder form of this drug was considered as a better alternative to the solution formulation for inhalation.

Using CS, a DPI formulation of ciprofloxacin (CFX) was investigated.[69] Spray dried microparticles (MPs) displayed improved aerosolization properties and the encapsulated drug showed high antimicrobial activity against *P. aeruginosa* and *Staphylococcus aureus*. The CS

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containing CFX showed better drug entrapment and longer dissolution times that led to prolonged release of drug from the MPs without significant toxic effects to lung epithelial cells. Lower cell viabilities caused by the MPs containing higher concentrations of CS were also noted; however, dextran containing CS particles were shown to be the least cytotoxic.

Glutaraldehyde crosslinked CS (**7**) microspheres loaded with antitubercular drugs, ofloxacin [52] and moxifloxacin,[70] have been investigated for pulmonary delivery. The ofloxacin loaded CS microspheres induced cellular uptake and prolonged release of drug from the microspheres. It was concluded that the lung delivery of drug loaded CS microspheres from a DPI formulation could improve the delivery efficiency of drug to alveolar macrophages and thereby reduce the duration of tuberculosis treatment with the unmodified, orally delivered drug by at least 6 months. With regards to the lung delivery of moxifloxacin, Ventura et al., prepared spray dried drug loaded glutaraldehyde crosslinked and non-crosslinked CS microspheres, which showed a significant initial burst release followed by a prolonged drug release from all crosslinked microspheres that contained the highest amount of glutaraldehyde in the formulation. Non-crosslinked microspheres rapidly swelled and released the dissolved free CS to interact with the cell by altering the biomembrane permeability; however, the crosslinked microspheres did not show this property. The crosslinking with glutaraldehyde was not required to retard absorption of drug through the pulmonary epithelium cells; however, significant prolonged release of drug was obtained from the crosslinked microspheres. Very recently, spray dried MPs of clarithromycin (a hydrophobic antibiotic) containing CS and leucine for pulmonary delivery from a DPI was reported.[71] The MPs showed excellent dispersibility (fine particle fraction, FPF, = 73%) due to the presence of leucine in the formulation, and exhibited slow release of drug due to the

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CS. The MPs did not induce toxicity on Calu-3 cells, indicating the safety of these particles for lung delivery.

The DPI formulation of another antitubercular drug, ethambutol dihydrochloride (EDH) encapsulated in CS NPs produced lower toxicity, higher potency and better permeation than the pure drug particles.[72] Three different cell lines i.e., A549, Calu-3, and NR8383 cells, were investigated and the cell viability of all of them showed promise. The CS coated EDH displayed a higher MIC (<1 μg/mL) compared with pure EDH (MIC 2 μg/mL) against *Mycobacterium bovis* and the CS contributed to the antimicrobial activities along with the EDH. The prepared DPI formulation of CS loaded EDH showed a sustained release of drug, suggesting that it could help to minimize the duration of treatment and the risk of developing multidrug resistance against tuberculosis compared to that of pure EDH. Spray dried inhalable powders containing isoniazid-loaded CS NPs showed sustained delivery of the drug to the lung.[73] An in vitro drug release study showed that the rate of isoniazid release from NPs was dependent on the amount of CS used in the formulation i.e., the drug released was decreased by increasing the CS concentration in the formulation, which indicted the prolonged release of drug from the NPs.

Among the antitubercular drugs, rifampicin has been the most extensively investigated for lung delivery. Nebulized rifampicin-loaded CS coated liposomes were found to show better mucoadhesive properties and lower toxicity (against A549 epithelial cells) compared with non-coated ones.[74] Using this drug, the development of MPs for drug delivery to the lungs from a DPI formulation by coating liposomes with CS-xanthan gum (XG) polyelectrolyte complexes, known as chitosomes, was also investigated.[75] Using a mixture of soy phosphatidylcholine and hydrogenated soy phosphatidylcholine in two different

concentrations, two groups of liposomes were prepared to encapsulate appropriate amounts of rifampicin. The obtained vesicles were then coated with different CS-XG weight ratios to prepare the desired liposomes and chitosomes. The nebulization and rheological properties of chitosomes were affected by the CS-XG weight ratio in the formulation. It was concluded that the CS-XG weight ratio of 1:0.5 (w/w) coating was able to greatly improve drug deposition (FPF = 37.8%) in comparison with the corresponding uncoated liposomes (FPF = 13%). The reason behind this has not been clearly demonstrated; however, it was suggested that the coating of liposomes with a polyelectrolyte complex at an appropriate ratio improved liposome resistance to aerosolization, which suggests that the CS has an impact on drug dispersion from DPI formulations. Recently, Garg et al.[76] demonstrated the application of spray dried inhalable CS NPs for sustained delivery of isoniazid and rifampicin. After administration, both drugs were detected in various organs (lungs, liver, spleen and kidney) until 24 hours post nebulization. It was concluded that the chemotherapeutic efficacy of a single dose of drug-loaded CS NPs was more effective against mycobacteria than the free drugs. Using rifampicin and isoniazid, spray dried CS based MPs with other excipients have also been investigated.[29] CS, guar gum, mannose and guar gum coated-CS were used for encapsulating the drugs. Guar gum containing spray dried particles showed a uniform size distribution with a smooth surface compared with mannose formulations; however, CS containing formulations exhibited the best surface morphology among the formulations, which could be related to the better mechanical strength of preformed CS NPs that resist particle squeezing during flash evaporation in the spray drying process. The drug release showed a biphasic pattern of release, i.e., an initial burst followed by a sustained release pattern due to the presence of CS in the formulation. An in vivo lung (mouse model) distribution study showed that the guar gum coated chitosan (GCS) formulations had a prolonged residence time at the target site and thereby improved the therapeutic application

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of drug with a significant reduction in systemic toxicity. The drug loaded GCS formulation resulted in almost 5-fold reduction of the number of bacilli compared with the control group. The preferential uptake of GCS NPs indicated the selective uptake capability of the mannose moieties present in guar gum to the specific cell surface of macrophages and that was an additional advantage in using guar gum in the formulation. It was concluded that the dual targeting DPI formulation containing GCS NPs could be a promising carrier for selective delivery of antitubercular drugs for efficient management of tuberculosis as well as other lung infections.

CS based rifampicin along with another potential antitubercular drug, rifabutin, was also investigated by Rohan et al.[60] Rifampicin and rifabutin loaded CS microsphere based DPI formulation systems were developed for the treatment of tuberculosis. An in vitro drug release study in simulated lung fluid (pH 7.4) showed a sustained release for 12 and 96 hours, respectively, for rifampicin and rifabutin microspheres, respectively. The drug release was dependent on both swelling of CS and the solubility of the drugs in the dissolution medium. The MPs were found to be taken up by a human macrophage cell line (U937), thus enabling targeting of *M. tuberculosis*. An in vivo acute toxicity study in rats revealed no significant local adverse effects. It was concluded that the MPs could be useful for developing an improved, targeted, and efficient system for the treatment of tuberculosis; however, extensive toxicological studies for repeated dose inhalation of these drugs is required to better assess their long term safety for pulmonary delivery.

Lung delivery of tobramycin embedded in PLGA NPs modified by CS to impart the desired surface property (i.e., positive charge) was investigated.[7] Initially, the PLGA based NPs were prepared and later coated with CS to obtain particles with the desired surface charge,

mucoadhesiveness, particle size and drug release properties. The surface of NPs of PLGA modified by positively charged CS showed a higher interaction with mucin compared to those of unmodified PLGA and PVA coated NPs. Therefore the adsorption of mucin onto the surface of CS modified PLGA NPs by shielding of NPs surface charge, was the dominant factor in the facilitated transport of NPs. Oritz et al.[77] developed dapsone-loaded CS microcapsules for deep lung delivery against *Pneumocystis carinii* pneumonia (PCP). The developed particles showed promising in vitro dispersion (FPF = 50%) and sustained drug release from the NPs. The particles were deposited into the deep lungs, and bronchioalveolar lavage fluid (BALF) analysis showed low cytotoxicity of the CS encapsulated drug compared with free drug. It was suggested that pulmonary administration of the drug loaded CS particles could be considered an effective and promising alternative for PCP treatment. Using CS as a carrier, clindamycin, a lincosamide antibiotic, was studied for in vitro lung delivery.[57] A DPI formulation of clindamycin hydrochloride loaded CS microspheres (particle size 1.7-1.8 μm) was prepared by spray drying and blended with a large lactose carrier (Inhalac 250). The formulation showed good flow properties due to the large carrier and prolonged clindamycin release occurred due to the presence of CS in the formulation.

Very recently, a CS based formulation of gentamicin, a widely used aminoglycoside antibiotic, was investigated for lung delivery with dual antimicrobial and antioxidant activity.[78] Due to the promising antioxidant[79] properties of fucoidan (a sulfated polysaccharide from seaweed) to scavenge the reactive oxygen species generated by gentamicin during treatment, Huang et al., used fucoidan with CS (CSF) to generate gentamicin encapsulated NPs and to study the prolonged release of gentamicin. The various electrostatic interactions between the negatively charged fucoidan and the positively charged CS and gentamicin enabled CSF NPs to encapsulate the drug efficiently. The cumulative

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concentration of the released drug indicated that almost 99% of the drug was released from CSF NPs in 72 hours. The intratracheal administration of drug-loaded CS/F NPs delayed the time to reach C_{max} in plasma, thereby reduced the risk of systemic toxicity caused by high gentamicin concentration in plasma. It was concluded that the intratracheal administration of gentamicin loaded CSF NPs into rat lungs improved antimicrobial efficacy with reduced systemic toxicity caused by high doses of intravenously injected gentamicin. The encouraging results from this study indicate that CSF based lung delivery of gentamicin shows promise for the treatment of pneumonia.

4.2. Proteins/peptides

As protein/peptides are susceptible to degradation by digestive enzymes after oral administration, lung delivery has been considered to be a more suitable delivery route. Therefore, proteins/peptides were the second most common drug class investigated for CS based lung drug delivery (Fig. 3, Table 1).

Using CS as a carrier, insulin has been investigated for pulmonary delivery.[48, 61, 62, 80] Yan et al. studied the absorption enhancing effects of CS NPs with various molecular weights on the pulmonary absorption of insulin in rats.[80] CS NPs with small size and surface properties i.e., positive zeta potential, were useful carriers for improving the pulmonary absorption of peptides and proteins. The surface positive zeta potential was considered to be the major contributing factor for the prolonged retention of drugs in lung tissues and penetration into the mucus layer. The absorption enhancing effects of CS NPs was dependent on the DD of CS. CS NPs with MWs from 35 to 168 kDa were found to be safe carriers for improving the pulmonary absorption of insulin without damaging lung tissues. Using a rat model, deep lung delivery of microencapsulated insulin-loaded CS NPs was shown to induce more pronounced

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and prolonged hypoglycemic effects compared with pure drug.[48] In another study, pulmonary delivery of insulin loaded CS NPs, with a small size and marked cationic charge was shown to improve the absorption of insulin. This was due to prolonged retention of NPs in lung tissue and penetration into the mucus layer without significant membrane damage to the lung tissues. It was suggested that the absorption enhancing effect of the NPs was affected by the DD of CS and was independent of the molecular weight. In addition, the CS NPs were found to have low toxicity. The lung delivery of insulin using CS modified PLGA nanospheres was also investigated[13] and it was shown that the NPs were eliminated from the lungs more slowly compared with unmodified PLGA NPs due to their mucoadhesive properties. This further supports the applicability of CS in lung drug delivery for controlled release. The in vitro delivery of dry powders of insulin containing CS prepared by spray drying showed promising dispersibility (FPF = 46%) with sustained insulin release over 60 hours.[61] Insulin loaded *N*-trimethyl CS (**2**) microspheres produced by a supercritical fluid technique also showed promising results.[62] The particle characteristics were maintained and the insulin structure was largely preserved in the microspheres even after storing the powders at 4°C for one year. However, no in vivo studies have been carried out.

The surface modification of CS and glycol CS (**3**) NPs with the surfactant Lipoid S100 for the pulmonary delivery of low molecular weight heparin (LMWH) has been investigated in a mouse model. [51] The administration of the surface modified NPs resulted in an increase in the systemic coagulation time, indicating that the NPs are promising carriers for pulmonary delivery of LMWH. Murata et al.[32] studied the lung delivery of elcatonin loaded in liposomes which were coated with low molecular weight CS oligosaccharide (oligo CS, MW~1000) and polyvinyl alcohol with a hydrophobic anchor (PVA-R) as a surface modifier. The PVA-R modification reduced interaction with A549 cells, whereas oligo-CS modification

electrostatically enhanced cellular interaction and thereby the therapeutic efficacy of elcatonin after pulmonary administration to rats was significantly enhanced and prolonged for 48 h. It was emphasised that the CS-modified liposomes adhered to lung tissues and caused opening of tight junctions, which enhanced the absorption of the drug. This study showed the superiority of oligo-CS over PVA in modifying drug loaded liposome NP surfaces for effective peptide drug delivery through the pulmonary route.

The lung delivery of insulin using CS modified PLGA nanospheres was investigated[13] and it was shown that the nanospheres were eliminated from the lungs more slowly compared with unmodified PLGA nanospheres due to their mucoadhesive properties. This further supports the applicability of CS in lung drug delivery for controlled release. The CS-modified PLGA nanocomposite particles exhibited enhanced pulmonary absorption of salmon calcitonin compared with non-modified PLGA nanocomposite particles via the lung delivery from a dry powder formulation.[8] The release and absorption of the salmon calcitonin encapsulated PLGA NPs coated with CS was controlled by the mucoadhesion and absorption enhancing effects of CS.[8] More than 40% of the CS modified PLGA NPs remained in the lungs 4 h after intratracheal administration into rat lungs. In another study, lung delivery of CS coated PLGA NPs containing palmitic acid-modified extendin-4 (Pal-Ex4) was investigated.[31] Promising outcomes such as higher absorption and delayed release of drug from the NPs were observed. Moreover, CS-coated PLGA NPs remained in the lungs for ~72 h, whereas most of the unmodified PLGA NPs had disappeared 8 h after administration. It is noteworthy that the hypoglycemic efficacy of inhaled CS coated Pal-Ex4 PLGA NPs was 3.1-fold greater than that of unmodified NPs.

4.3. Anticancer drugs

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The use of CS as a surface modifier for lung delivery of anticancer drugs has not been extensively explored compared with other drug classes (only 17%, Fig. 3). CS-modified PLGA NPs containing paclitaxel delivered into rat lungs showed significantly increased uptake and cytotoxicity against a lung cancer cell line (A549). [81] A lung-specific increase in the distribution of paclitaxel was observed for CS-modified NPs compared with unmodified drug. It was not fully clear why the lung specifically accumulated the drug; however, the formation of large NP agglomerates in contact with plasma proteins during circulation, followed by enhanced entrapment of the formed agglomerates by the lung capillaries, was thought to be the cause. In addition, it was also demonstrated that the electrostatic interaction between the positively charged NP surfaces due to CS coating and negatively charged endothelium of lung tumour vasculature was also responsible for the lung-specific drug accumulation. This is an encouraging result for delivery of drug into the target organ for the treatment of lung cancer. Recently, docetaxel-loaded CS microspheres, prepared by glutaraldehyde crosslinking (**7**) were shown to release the drug to a maximum extent in the target tissue i.e., lung, confirmed by in vivo studies in a mouse model. In vitro release indicated that the CS coated drug microspheres had a well-sustained release profile (70% drug released in 19 days). [54] Using CS coating technology, lung delivery of CS-coated PLGA NPs containing 2-methoxyestradiol (2-ME) showed good dispersibility, high respirable fraction and a sustained release profile.[59] It was shown that the NPs were suitable for lung delivery and significantly enhanced cytotoxicity of 2-ME without noticeable inflammation in rat lungs, suggesting that inhaled 2-ME CS NPs have great potential for targeted, highly effective and safe treatment of lung cancer.

Cisplatin, another widely used anticancer drug has been investigated for lung delivery.[12, 82] Positively charged CS coated cisplatin micro/NPs showed cytotoxicity similar to or lower

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than that of the pure drug on P388 murine and A2780 human cells. The NPs with the smallest size and the lowest positive zeta potential due to the surface CS showed more activity than that of uncoated cisplatin on A549 human cells. Singh et al. [12] studied the lung delivery of CS coated cisplatin and achieved better efficacy against lung cancer by using CS coated cisplatin microspheres compared with the drug alone. The lung delivery helped localize the drug in the lungs, and provided sustained action. The drug release from CS microspheres showed a biphasic pattern with an initial burst effect, followed by a subsequent slow release. Cisplatin loaded CS microspheres showed higher cytotoxic effects on A549 human lung cancer cells and a higher IC_{50} value compared with the free drug. Additionally, the drug loaded CS microparticulate DPI system was stable for 6 months at 25°/60% RH and at 40°/75% RH, without significant changes in drug content, except reducing the FPF.

4.4. Anti-asthmatic drugs

Inhaled formulations of anti-asthmatic drugs have been proven clinically effective and several drugs in DPI or pMDI (pressurized metered dose inhaler) forms are available on the market. CS based formulations of anti-asthmatic drugs are yet to be approved for lung delivery; however, some drugs such as salbutamol sulfate,[57, 83] theophylline,[18] terbutaline sulfate and beclomethasone dipropionate[30] have been investigated for sustained release delivery from CS based nano/microparticles.

The most useful and available anti-asthmatic drug, salbutamol sulfate, has been formulated using CS for sustained release delivery.[83] The spray dried CS-salbutamol sulfate microparticles showed increased deposition in the target sites with FPF of 55%-65%, which is much higher than that of large, lactose carrier-based formulations (FPF 11.2%). Additionally, the CS based salbutamol sulfate produced sustained release of drug from the

microspheres. It was suggested that the CS-modified spray-dried powders not only enhanced the dispersibility and lung deposition, but also produced prolonged release of drug; however, the mechanism of how CS enhances the dispersibility was not demonstrated. Prior to this study, Kamble et al. used the same salbutamol sulfate-encapsulated CS microspheres for inhalation purposes.[57] The CS microspheres were prepared by spray drying with or without a crosslinking agent, citric acid. The microspheres showed excellent flow properties and the crosslinking agent was found to decrease the entrapment efficiency and in vitro sustained release of drug. This effect might be due to the reduction in swelling ability of the CS in the microspheres because of the high concentration of citric acid increasing the rigidity of microspheres. Recently, Zhang et al. studied the DPI formulation of CS based theophylline.[18] Microspheres were prepared by spray drying theophylline, carboxymethyl CS (**4**) and β-cyclodextrin, which produced irregular spherical shapes with or without smooth surfaces. In addition, the cytotoxicity of the microspheres was concentration dependent and no toxicity to liver, kidneys or haemoglobin was observed at low concentrations (<0.1172 mg/mL). An in vivo implant experiment in Sprague-Dawley rats demonstrated that the particles were biocompatible.

CS based sustained release delivery of beclomethasone dipropionate[55] and terbutaline sulfate [58] from DPI formulations have also been investigated. Using different molecular weights of CS (separately or different combinations), the spray dried microparticles of hydrophobic drug beclomethasone dipropionate [55] were prepared. With increasing molecular weight of CS, the FPF of drug decreased; however, the time for 100% drug release was increased. The same research group later studied the sustained release of the combination of hydrophilic (terbutaline sulfate) and hydrophobic (beclomethasone dipropionate) drugs from CS based

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microparticles for pulmonary delivery. Increased CS molecular weight was associated with increased duration of drug release i.e., favoured sustained release of drugs.

Large porous particles have a significant effect on the lung delivery of drugs[84] and intratracheal delivery (in rat) of porous particles of CS containing budesonide from a DPI formulation prepared by spray drying, showed an extended half-life compared with a conventional formulation and with a fourfold improvement in local and systemic bioavailability. Similar anti-inflammatory activity of budesonide and developed formulations has been observed.[85] Improved aerosolization efficiency was also reported with PLGA and soy lecithin (PC) NPs (PLGA-PC) from a DPI formulation containing large porous particles of CS microparticles crosslinked with TPP and dextran sulfate DXT).[86] The prepared porous CS carrier particles (CS-TPP-DXT) exhibited desired aerosolization characteristics and physicochemical robustness due to their electrostatic properties. Due to the high negative charge, the carrier particles were limited to adsorb only cationic nanoparticles, which was the drawback of the prepared carrier.

4.5 Other drugs

A DPI formulation of itraconazole loaded CS NPs crosslinked with TPP has also been reported. [87] A CS based DPI formulation of carvedilol containing poly(ethylene-co-vinyl acetate) (PEVA) NPs coated with CS for lung delivery has been investigated. [88] The NPs were spray dried with lactose or mannitol as carriers. The results revealed that a high amount of PEVA NPs was associated with mucin, confirming the enhanced mucoadhesion of NPs coated with CS compared with that of uncoated NPs. The in vitro aerosolization behaviour

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indicated that the mannitol-based formulation was found to have better flow properties and higher FPF.

Recently, Yang et al. [89] extended the application of CS and CS-oligo to a DPI formulation of *fanhuncaoin*, a newly discovered anti-inflammatory drug isolated from a Chinese herb. Both CS and CS-oligosaccharide showed excellent dispersibility enhancement of the spray dried inhalable powder. Powder formulations without CS led to the poorest flowability and the formed particles appeared fused with each other. It was suggested that CS modified the surface of the powder particles and contributed to decreased interparticulate cohesion forces and thereby improved powder dispersibility.

4.6. Genes/nucleotides

Pulmonary delivery of genes/peptide has become an emerging are of research a list of investigated genes/nucleotides has been presented in Table 2. Lung delivery of plasmid DNA (pDNA) from a DPI formulation has been studied.[90] Spray dried pDNA containing CS and its soluble derivative *N*-trimethyl CS (**2**) were prepared. Formulations of both CS and **2** showed significantly greater emitted dose (> 90%) and in vitro pulmonary deposition (FPF > 35%) of dry powder dispersions compared with powders without CS (emitted dose 57%; FPF 12%). Both CS and **2** enhanced the level of reporter gene expression to human lung carcinoma cells.

Using a supercritical fluid technique, dry powders of CS-pDNA complexes were prepared for lung delivery. [91, 92] In vivo pulmonary transfection studies in mice indicated that the powders had higher transfection potency than the aqueous solutions containing the same amount of DNA. The removal of CS from the formulation led to extensive degradation of DNA during manufacturing and storage. Therefore, it was concluded that the CS improved the stability of

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pDNA powder during manufacturing and storage of the formulations.[63] CS and CS coated PLGA NPs containing BSA and DNA showed highest burst release (43%) within two days and more than 80% sustained release in 3 weeks; however, CS-PLGA NPs were more stable (5 days at 37ºC) than that of CS NPs.[46]

Okamoto et al. [93] studied CS-β-interferon gene complex (pCMV-Muβ) powders as a non-viral vector for intratracheal administration to compare their gene expression and therapeutic efficacy using mice burdened with pulmonary metastases. A luciferase expression system revealed that the genes expressed in both normal and tumorous tissues and the intratracheal powder resulted in higher expression than the intravenous or intratracheal solution formulations. The intratracheal administration of powder pCMV-Muβ at a dose of 1 μg significantly extended the mean survival time compared with untreated control. The results of this study indicated a superior therapeutic effect of CS/pCMV-Muβ powders against a murine model of lung metastasis with respect to the untreated drug. It was concluded that therapeutic gene powders for pulmonary delivery are promising for gene therapy to treat lung cancer. Although the effects of CS in the formulation are not fully clear; it did improve the stability of the powder formulation.

Stable CS NPs encapsulating siRNA were prepared using an ionic crosslinking technique for lung delivery and were found as suitable carriers for delivery of siRNA using a jet nebuliser. [94] Very recently, Jeong et al. [95] also found promising activity in terms of cellular uptake and gene silencing of CS based siRNA NPs after intratracheal administration in a mouse model. Intratracheal administration of CS-coated PLGA based nanoplexes with antisense 2'-O-Me RNA (OMR), showed higher cellular OMR uptake compared with OMR alone.[96] The results showed no remarkable changes in lung physiological parameters after lung delivery of this

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drug and suggested appreciable local tolerability of the OMR-nanoplex formulation. Here CS facilitated intracellular uptake of the OMR-nanoplex. Regnstroem et al., studied the gene expression profiles of CS alone or CS formulated luciferase reporter plasmid (CS-pLuc) in pulmonary delivery (intratracheal administration) in mouse lungs.[97] CS-pLuc was found to upregulate genes that protect the cell from oxidative stress and inflammation, such as heme oxygenase-1 and catalase. In addition, CS-pLuc activated genes involved in reaction to stress, such as DNA damage repair.

4.7.Vaccines

Administration of a vaccine to the lungs avoids the use of needles, as many people suffer from injection phobia. Furthermore, pulmonary vaccination also induces a mucosal immune response in addition to the systemic immune response obtained by injection. Such a local response may increase the effectiveness of vaccination against pathogens. Lung delivery of vaccines has been studied for decades; however, no pulmonary vaccine products are commercially available as yet. Although lung delivery of vaccines is not yet established, large numbers of vaccine delivery formulations (Table 3) have been investigated.[98-100] In vaccine formulation, CS has been used as an adjuvant due to its nontoxic and mucoadhesive properties and it has been found to boost antibody responses to intranasal delivered influenza vaccine dry powder formulations.[41] CS has also been reported to directly activate the immune system due to its cationic nature[28] when used as a vaccine adjuvant. CS-derived polymer NPs protected DNA vaccines against degradation by DNAse, resulting in enhanced immunogenicity of a DNA vaccine against hepatitis B virus.[101] The CS derivatives **9**-**11** were prepared, respectively, via ammonium persulfate-initiated free radical polymerization with methylmethacrylate, *N*-trimethylammoniumethylmethacrylate, and *N*-dimethylammoniumethylmethacrylate as monomers, respectively.

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The lung delivery of DNA vaccines against tuberculosis was found to be advantageous compared with intra muscle immunization, and the increased immunogenicity was achieved by delivery of this DNA encapsulated in CS NPs.[27] Using an HLA-A2 transgenic mouse model, the pulmonary delivery of DNA plasmid encoding eight HLA-A*0201-restricted T-cell epitopes from *M. tuberculosis* formulated in CS NPs was studied.[27] Lung delivery of the DNA plasmid incorporated in CS NPs was found to induce increased levels of IFN-γ secretion compared with pulmonary delivery of plasmid in solution for intramuscular immunization. Pulmonary delivery of CS complexed with *M. tuberculosis* DNA vaccine (mut-T-epitope DNA vaccine, pHSP65pep) enhanced the mucosal immunity.[102] CS was found to significantly increase pulmonary CD4+ IFN-γ+ and CD8+ IFN-γ+ T cell frequency induced by pHSP65 pep, which was much higher than that of the splenic counterpart. The CS-pHSP65pep complex produced better protection compared with the vaccine without CS.

Recently, the uptake and immune response of adjuvant Pam3Cys (Toll-like receptor-1/2 agonist) coupled with CS-DNA NPs were explored by using a three-dimensional human epithelial cell culture model.[103] CS-based DNA NPs delivery into lung cells was shown to induce antigen-specific immunity. In addition, the Pam3Cys adjuvanted CS-DNA NPs significantly enhanced the Th1 associated immune response due to elevated IL-8 and TNF-α levels. The overall results were promising for pulmonary DNA vaccination against intracellular pathogens (*M. tuberculosis* or influenza virus). Amidi et al.[104] studied the potential of *N*-trimethyl CS (**2**) microparticles for pulmonary delivery of diphtheria toxoid (DT). The pulmonary immunization with DT-loadedmicroparticles of **2**, prepared by supercritical fluid extraction, showed a strong immunological response reflected by inducing IgM, IgG, IgG subclasses (IgG1 and IgG2) antibody levels as well as neutralizing antibody titers comparable to, or significantly higher than, those observed after subcutaneous

administration of conventional alum-adsorbed DT antigen. In addition, CS based DT microparticles induced detectable pulmonary secretory IgA levels. It was concluded that the pulmonary delivery of CS based DT microparticles is a potent new delivery system against diphtheria.

4.8 . Chitosan derivatives and pulmonary drug delivery

With a view to achieving better physicochemical properties (i.e., improved solubility), low toxicity and improved biodegradability, various types of chemical modifications in CS structure have been studied. The applicability of chemically modified CS/ CS derivatives for lung delivery of different drugs is discussed below.

Very recently, gentamicin (GM) encapsulated CS/fucoidan NPs, as demonstrated in Fig.4, for lung delivery against *Klebsiella pneumoniae* were developed [78] and intratracheal administration of drug loaded CS/fucoidan NPs in rats presented a superior antimicrobial efficacy with eliminated systemic toxicity. It was demonstrated that the CS contributed the first line antimicrobial function before releasing sufficient amount of drug from the NPs and thus exerted a synergistic antimicrobial effect. However, the contributions of fucoidan to the NPs physicochemical properties, dispersibility, drug release from NPs and antimicrobial activities were not addressed.

TPP crosslinked CS NPs with added PEG 1000 were prepared for lung delivery from pMDIs.[50] PEG was used in the formation of NPs as it acts as a polymeric surfactant that helps reduce the cohesive interactive forces among particles suspended in a suitable solvent. Due to the presence of CS, particles were positively charged and non-aggregated at the pH of the airways. Crosslinked CS-PEG 1000 NPs showed greatest dispersibility and physical stability

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in HFA-227 due to the positive charge of CS. The results demonstrated the superiority in terms of stability of CS based NPs in pMDI formulations compared with that of formulations without CS. Makhlof et al. [34] coupled glycol CS (**3**) with thioglycolic acid mediated by the water-soluble carbodiimide EDAC to give conjugate **12** and evaluated it for the pulmonary delivery of peptide calcitonin. Drug loaded NPs of **3** and **12** were prepared by an ionic gelation technique. Following intratracheal administration to rat lungs, NPs of **12** showed a two-fold increase in mucoadhesion to lung tissue compared with non-thiolated NPs. Calcitonin-loaded NPs of **3** and **12** resulted in a prolonging of the hypocalcemic effect for at least 12 and 24 hours, respectively. A pulmonary toxicity assay revealed the biocompatibility of the NP formulations with lung tissue. These findings suggest that both **3** and its thiolated derivative **12** are promising and safe carriers for pulmonary delivery of peptide drugs such as calcitonin. Spray dried levofloxacin encapsulated in hydrophobically modified CS with octanoyl groups (**8**) was found to enhance dispersibility of drug from a DPI formulation [68]. In vitro cytotoxicity evaluation using the MTT assay performed on the A549 cell line confirmed that the formulations were non-cytotoxic. The modified CS showed significantly lower MIC (4-fold) than that of commercially available CS against *P. aeruginosa*. Drug loaded modified CS microparticles showed higher FPF (52%) compared to that of non-modified CS (FPF 20%) and thus **8** was considered a highly efficient dispersibility enhancer for use in the DPI formulation.

Jin et al. [105] synthesised a urocanic acid conjugate of CS (**13**) via EDAC mediated coupling, which was complexed with the protein PTEN (phosphatase and tensin homolog deleted on chromosome 10) and delivered to the lungs of K-ras^{LAI} mice through a nose-only inhalation system. The **13**- PTEN complex significantly suppressed the development of lung tumor through the formation of a nuclear complex between PTEN and p53, by suppressing Akt-

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related signals as well as cell cycle regulation. It was suggested that **13** is a promising aerosol delivery vehicle with little toxicity and enhanced gene transfection efficiency and **13**-mediated PTEN delivery may be compatible with non-invasive in vivo gene therapy for prevention and treatment of lung cancer. Luo et al.[106] reported the application of a guanidinylated CS-salbutamol conjugate (**14**) as a carrier for lung delivery of siRNA targeting the β_2-adrenoceptor. CS was firstly coupled with salbutamol, a β_2-adrenoceptor agonist, in the presence of epichlorohydrin and the resulting product was then guanidinylated with aminoiminomethanesulfonic acid increase non-specific cellular uptake. The final conjugate **14** was substituted with approximately 3.5% salbutamol and 8.2% guanidino groups. Conjugate **14** facilitated targeted gene delivery into the mouse lungsand was shown to decrease the cytotoxicity against HEK293 cells and enhance gene-silencing efficiency compared with CS. It was thus suggested that **14** is a promising carrier for siRNA delivery for the treatment of viral respiratory infections.

Very recently, Jeong et al.[95] synthesised cell-penetrating peptide **-**modified CSs (**15**-**17**) by conjugating a peptide consisting of nine arginine units (R_9) and a variable spacer arm of glycine units (G_n) via the C-terminal carboxylate to the amine of CS via the carbodiimide method. The degree of substitution was quite low due to the poor solubility of the R_9G_n peptides, with only an average of two peptides conjugated per CS molecule. The conjugates were complexed with siRNA to investigate cellular uptake and gene silencing. In a mouse model, intratracheal administration of NPs revealed that the conjugate with the longer spacer arm (**17**, R_9G_{10}) was more effective in delivering genes compared with unmodified CS.

El-Sherbiny and Smyth[107]grafted mPEG5000 onto both CS (PEG-g-CS, **18**) and its *N*-phthaloyl derivative (PEG-g-NPHCS, **19**) via EDAC-mediated coupling and prepared

swellable microparticles using a cryomilling process to investigate the suitability for lung delivery of a model drug, sodium fluorescein. The drug loaded microspheres of **19** showed higher values of cumulative drug release compared with microspheres of **18**. The reason behind this behaviour was the presence of the hydrophobic phthaloyl group in **19** which retarded the entrance of fluid into the matrix and consequently led to the slow release of drug. Subsequently copolymer **19** was self-assembled into bovine serum albumin (BSA)-loaded NPs. The NPs were encapsulated in respirable sodium alginate semi-interpenetrating polymeric network (IPN) hydrogel microspheres, prepared by spray drying, for controlled release pulmonary drug delivery. The FPF obtained was 31.5% with a slow sustained release of BSA up to 4 days. The enzymatic degradation of microparticles in 0.2 mg/mL lysozyme occurred within the first 2 hours; however, the rate of degradation was reduced with increasing percentage of **19** in the microsphere. The results from both studies suggest that the microspheres of synthesised PEG grafted copolymer with CS could be suitable for inhalation and drug delivery to the deep lungs. These encouraging outcomes inspired El-Sherbiny and Smyth[108] to use **19** for lung delivery of a widely used antibiotic, ciprofloxacin. Ciprofloxacin-loaded and blank self-assembled NPs were prepared using a sonication technique and later the NPs were encapsulated into respirable alginate micro hydrogel particles (Fig. 5) and intratracheally delivered into rats by insufflation. The swellable particles showed lower ciprofloxacin levels in plasma compared with the control group (a mixture of large carrier lactose with micronized ciprofloxacin); however, produced higher drug concentration in lung for more than 7 hours, which is indicative of prolonged drug release from the formulation.

Recently, Berezin and Skorik[109] conjugated CS with isoniazid via two different methods. Firstly, CS was reacted with either epichlorohydrin or acrylic acid and the resulting products were coupled with isoniazid to give conjugates **20** and **22**. The acrylic acid method was

preferred because it gave a higher degree of isoniazid substitution (up to 0.39). Due to their poor water solubility, **20** and **22** were phosphorylated to give conjugates **21** and **23**. The conjugates were found to show high biocompatibility and relatively low toxicity.[109] The in vitro antitubercular activity of the conjugates against *M. tuberculosis* H37Rv was lower compared with unmodified drug. It was suggested that the decrease in antitubercular activity was probably caused by limited availability of the bound drug for the antimicrobial action or incomplete cleavage of the strong C-N bond between drug and CS. The authors did not investigate the impact of the lung delivery of the synthesised conjugate. Very recently, the potential of a water-soluble CS-leucine conjugate (**5**), as a matrix for enhancing the dispersibility of NPs from a nanoparticulate DPI formulation was demonstrated.[11] The surface hydrophobicity achieved by the hydrophobic domain of leucine (Fig. 6 A) and glutaraldehyde crosslinks formed on the NP surface was found to contribute to the enhanced dispersion (Fig. 6 C; FPD 0.31 mg) of the conjugated CS NPs. The promising rapid and prolonged drug release (Fig. 6 B) from leucine-conjugated NPs was associated with the nature of the cross-links as well as the amide groups that provided H-bonding with water thus influenced the drug release by swelling the nanoparticles. Previously, the low toxicity of the conjugate NPs for pulmonary delivery was demonstrated.[43]

Conclusions and future directions

Research in developing CS based lung drug delivery is progressing and has achieved considerable success so far. CS is universally recognised as a non-toxic, biocompatible and biodegradable polymer and has advantages over other biodegradable polymers as it is mucoadhesive, an absorption and dispersibility enhancer, immunogenic, and can encapsulate both hydrophilic and hydrophobic therapeutic agents. In this review, the advances in developing CS based inhaled formulations of various drugs investigated so far have been

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reviewed. Due to the enhanced drug delivery efficiency, reduced unwanted adverse effects, increased solubility, improved surface hydrophilicity/hydrophobicity to encapsulate various drugs, and prolonged drug release, the conjugates of CS with drugs or other excipients, showed significant potential for extensive investigation for clinical applications. Appropriate engineering of designed nanoparticles (chemistry, size and surface charge) for in vivo lung drug delivery is required to optimise the performance. To date, CS structure modification has mostly been carried out at the amino groups at the C2 position (Figure 2) and the prepared particles have not been fully investigated for toxicity, drug accumulation and release. In future, more derivatives of CS with modifications at C6 or both the C2 and C6 sites will be prepared and their manufacturing technologies, physicochemical properties, extent of sustained drug release, and importantly, the toxicities in human organs will be studied. Most of the major cross-linkers, surfactants and certain grafting agents used in the preparation of CS nanoparticles are not free from toxicity, which is essential to account for long term exposure. The biodegradability, biodistribution and biocompatibility of CS and especially its derivatives are not fully understood and therefore, in future, careful investigations are required. Due to the characteristics of its chemical structure, cross-linkers are often required; however, the optimum cross-linker(s) for the formation of NPs to needs be identified. Currently, TPP is widely used as a less toxic cross-linker, but more studies are required to search for better cross-linkers with reduced or no toxicity. Few in vivo studies have been carried out so far and therefore, a deeper knowledge on the in vitro/in vivo toxicity of both CS and its derivatives for prolonged release drug delivery needs to be investigated in detail. To strengthen the polymer based nanoparticulate lung delivery of various drugs, multidisciplinary researchers, i.e., polymer scientists, biologists, immunologists, pharmacists, and medical practitioners need to work together. Translation of information from lung delivery to animals (most frequently used mouse and rat models) to humans is very difficult

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because of the significant differences between the physiology of the respiratory tract of animals and humans. In addition, the administration routes used for animal models are not suitable for human use. It is extremely difficult to evaluate and justify the lung delivery efficiency of the prepared formulations before considering them for clinical studies. Therefore, the selection of appropriate lung drug delivery models, which will result in achieving their translation into clinical trials is essential. Future research will focus on the appropriateness of the inhaled drug delivery technology such as design of polymer conjugate with reduced toxicity, optimization of NP fabrication and cross-linking, improved solubility of hydrophobic drugs, and the capability of accommodating both hydrophilic and hydrophobic drugs. CS based nanoparticulate drug delivery will create a promising roadmap to deliver a large number of therapeutic agents into deep lungs for both local and systemic effects.

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Table 1: Drug encapsulated CS micro/nanoparticles investigated for lung delivery

Drugs	Carrier	Formulation/ delivery	Main findings	Ref.
Proteins/ peptide				
Insulin	CS+ mannitol	Spray dried drug loaded microparticles; in vitro drug release	Reduced FPF compared to other formulations	[61]
Insulin	CS	Microencapsulated insulin loaded CS NP; rat model	Improved absorption for systemic action	[48, 80]
Insulin	CS; TMCS	Supercriticaly (SCF) produced insulin coated CS microparticles	SCF drying was a promising technique to prepare inhalable insulin loaded CS microparticles	[62]
Palmitic acid modified Extendin 4 (Pal-Ex4)	GCS + PLGA	Nebulization of NPs in rats	Improved cellular uptake; delayed release; hypoglycemic efficacy was 3.1-fold higher than that of unmodified drug	[31]
Elcatonin	CS + liposome	Drug loaded liposome NPs coated with CS	Enhanced absorption and therapeutic action; prolonged drug release	[32]
Calcitonin (hormone)	CS +PLGA	CS-modified PLGA nanospheres loaded with drug	enhanced the absorption by opening the intercellular tight junctions; sustained release profile	[8, 24]
Heparin (LMW)	CS and GCS	Intratracheal administration of NP in mice	Increased blood coagulation time with respect to pure heparin	[51]
Octreotide	CS and TMC	Intra-tracheal instillation in rats	Permeation enhancer; sustained release; low toxicity	[25]
Antibacterial/ antitubercular				
Isoniazid	CS + lactose+ mannitol + maltodextrin	Drug-loaded CS NPs spray dried with lactose, mannitol and maltodextrin alone or with leucine.	CS based drug decreased activity against *M.avium;* leucine increased dispersibility (FPF 45%)	[73]
Levofloxacin	CS	In vitro lung	Improved dispersion,	[53]

		deposition from DPI, release and antimicrobial activities	antimicrobial activities with controlled release profile	
Ciprofloxacin	CS, CS + DX	Spray dried drug loaded CS or CS+DX inhalable microparticles	Promising activity against *P.aeruginosa* and *S. aureus*; controlled drug release; safe on lung epithelial cells	69
Rifampicin + isoniazid	CS	Spray dried drug loaded CS NPs for nebulization in rat	Encapsulated drug was more effective against the mycobacterium than free drug; controlled release	76
Rifampicin + isoniazid	CS + guar gum	CS coated and guar gum coated CS particles, spray dried with mannitol and leucine	improved therapeutic activity against tuberculosis with reduced cytotoxicity	29
Rifampicin	CS +XG + liposome (chitosome)	Freeze dried drug loaded liposome coated with CS and XG microparticles	improved in vitro drug deposition	75
Tobramycin	CS + PLGA	Intratracheal administration in rats for tuberculosis	Modified surface influenced better aerosolization and deposition	7
Ethambutol	CS	In vitro lung delivery for tuberculosis	lower toxicity, higher potency and better permeation than the pure drug	72
Dapsone	CS	Dapsone-loaded CS microcapsules for in vitro lung delivery	High FPF (50%) and low toxicity; effective against *Pneumocystis carinii* pneumonia	77
Ofloxacin	CS	Drug loaded CS microsphere	Improved drug uptake; reduced the duration to cure tuberculosis	52
Moxifloxacin	CS	Spray dried drug loaded CS microsphere	Sustained drug release up to 4 days	70
Rifampicin + Rifabutin	CS + lactose	Spray dried drug loaded CS particles blended with lactose carriers	Improved, targeted and efficient delivery system for the treatment of tuberculosis	60
Gentamicin	CS + fucoidan (F)	Drug loaded NPs against *K. pneumoniae;* intra-tracheal rat model	Superior antimicrobial capabilities with reduced toxicity; sustained release profile	78
Clarithromycin	CS + leucine	Spray dried particles	Improved dispersibility;	71

		for in vitro delivery	no toxicity on Calu-3 cells observed	
Clindamycin	CS + lactose	Spray dried powders for vitro drug dispersion	Improved dispersion with sustained release	57
Vancomycin	CS +liposomes (chitosomes)	Drug loaded liposomes modified with CS; nebulized by rat	Improved drug transport, tissue uptake and systemic circulation	110
Anticancer				
Paclitaxel	CS + PLGA	CS modified paclitaxel loaded PLGA NPs delivery into mice lung	Increased uptake and cytotoxicity against lung cancer cell line (A549)	81
Docetaxel	CS	Drug-loaded CS microspheres; in vitro and in vivo studies	Sustained release of DTX; increased the accumulation of drug in target sites	54
Cisplatin	CS	NPs of anionic cisplatin -alginate complex with cationic CS	Particles with lowest positive zeta potential were more active than the pure drug on A549 human cells	82
Cisplatin	CS	Drug loaded CS microspheres; emulsification method; in vitro test	Higher IC_{50} against A549 and HOP 62 lung cancer cell lines; slow release	12
Methoxy-estradiol	PLGA + CS	Spray dried NPS; Lung delivery in rat model	Improved dispersibility with sustained release; enhanced cytotoxicity without inflammation; effective and safe for lung cancer	59
Anti-asthma				
Salbutamol sulfate	CS + lactose	Spray dried CS microspheres of drug and lactose based dry powder	Delayed drug release	83
Theophylline	CM-CS + β-cyclodextrin	Spray dried microspheres for lung delivery	Biocompatible; potential carrier for lung drug delivery	18
Terbutaline sulfate + Beclomethasone dipropionate	CS + leucine	In vitro dispersibility and release studies of spray dried drug	Improved dispersibility with sustained release profile	30
Beclomethasone dipropionate	CS + lactose + leucine	In vitro dispersibility and release studies of spray dried powder	Delayed drug release	56

Budesonide	Spray dried drug coated large porous CS particles	In-vitro and rat model	Improved half life and bioavailability	85
Others				
Itraconazole (antifungal)	CS + lactose + mannitol + leucine	In vitro drug dispersion	Improved the aerosolization; sustained release	87
Carvedilol (anti-hypertensive)	CS + PEVA+ mannitol/ lactose	Drug loaded PEVA NPs coated with CS and spray dried with lactose/ mannitol	Mucoadhesive and controlled release; higher FPF (58.8%)	88

Note: DX: Dextran; XG: Xanthan gum; GCS: glycol CS (**3**); CM CS: carboxymethyl CS (**4**); PEVA: poly(ethylene-co-vinyl acetate); TMCS: *N*-trimethyl chitosan (**2**).

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Table 2: CS based lung delivery of genes

Drug molecule	Formulation	In vivo model	Main findings	Ref.
pDNA	Spray dried CS coated pDNA NPs	In vitro lung delivery	CS improved dispersibility; enhanced level of reporter gene expression	[90]
DNA	CS + PLGA	Nebulization of NPs in rats	Higher cytocompatibility with lung cells; sustained release	[46]
pDNA	CS	Microparticles delivered in mouse	Increased the luciferase activity and stability of pDNA powder	[91, 92]
Antisense 2'-O-Me RNA (OMR)	CS coated PLGA NPs	Nanoplexes with antisense 2'-O-Me RNA (OMR); rat model	Produced higher cellular OMR uptake; good local tolerability	[96]
β-interferon	CS + mannitol	CS-INF complex; intra-tracheal mouse model	Promising gene therapy to treat lung cancer or metastasis	[93]
luciferase reporter plasmid (CS-pLuc)	CS	intratracheally administered into Balb/c mice	Activated genes involved in reaction to stress, such as DNA damage repair	[97]
siRNA	CS	siRNA encapsulated crosslinked CS NPs for nebulization	High cell viability at the highest CS conc.; potential for siRNA delivery to the lungs	[94]
siRNA	CS+ oligopeptide	Intratracheal administration of NPs in mouce model	Modified CS NPs were more effective in delivering genes compared with pure CS	[95]

pDNA: plasmid DNA

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Table 3: Nanoparticles of CS encapsulated lung vaccine delivery

Drug molecule	Formulation	In vivo model	Main findings	Ref.
Diphtheria toxoid	CS	DPI formulation delivery in guinea pig	induced strong immune response (induction of IgA antibody)	104
The pHSP65-pep DNA complexed with CS	CS	Intranasal, mice model	induced specific mucosal Th1 response and SIgA level; better than pure vaccine	102
DNA vaccine (pVAX(HBc)DNA)	CS	Intratracheal administration in mouse model	CS NPs were safe for effective DNA delivery	101
DNA vaccine (HLA-A*0201-restricted T-cell)	CS	DNA vaccine complexed with CS; mouse model	Increased immunogenicity against tuberculosis	27
pDNA	CS	CS coated DNA NPs with pam3Cys; ex vivo cell model	Elevated IL-8 and TNF-α level	103

Table 4: Drug encapsulated particles of CS derivatives investigated for lung delivery

Formulation	Drug molecule	In vivo model	Main findings	Ref.
CS or **8**	Levofloxacin	Spray dried drug + CS or **8**	hydrophobic **8** enhanced dispersibility of NP and noncytoxic	[68]
14	siRNA	NPs nebulised in mice	Decreased cytotoxicity; enhanced the transfection efficiency	[106]
12	Calcitonin	Intratracheal administration to rat	Enhanced mucoadhesion; pronounced hypocalcemic effect	[34]
18, **19**	Sodium fluorescein (model drug)	Microparticles for in vitro delivery	Sustained drug release	[107]
19	BSA	BSA loaded alginate IPN hydrogel microspheres; in vitro delivery	Sustained drug release	[111]
19	Ciprofloxacin	Dry powder of NPs delivered into rat lungs	Higher drug concentration in the lungs; sustained released profile	[108]
13	PTEN	Aerosols of UAC-PTEN were delivered into K-rasLA1 lung cancer mice model	Suppressed lung tumor, Akt-related signals, and cell cycle regulation	[105]
15-**17**	siRNA	siRNA coated CS complex NPs delivered into mice lung	Enhanced cellular uptake and the gene silencing efficiency CS/siRNA NPs	[95]
5	Diltiazem hydrochloride (DH)	DH encapsulated CS and conjugated CS NPs	Low toxicity, enhanced dispersibility, rapid and prolonged drug release	[11, 43]

PHCS: Phthalolyl CS; NPHCS: *N*-Phthalolyl CS; PTEN: Phosphatase and tension homolog deleted on chromosome 10.

Figures

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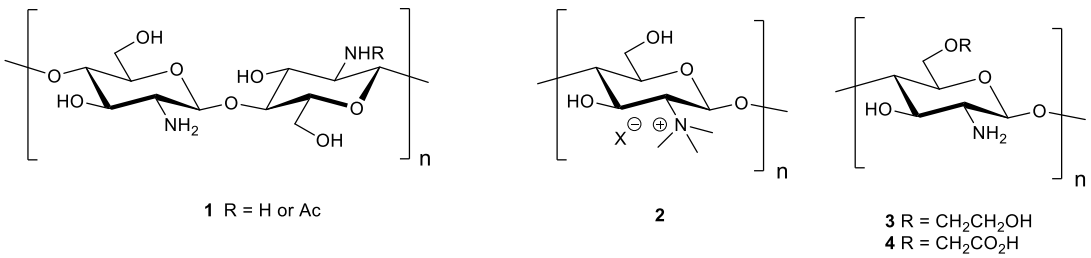

Fig. 1 The structure of the repeating units of chitosan (CS) **1** and some derivatives, *N*-trimethyl chitosan (**2**), glycol chitosan (**3**) and carboxymethyl chitosan (**4**). Note that CS preparations contain varying amounts of *N*-acetyl groups but for simplicity CS derivatives will be depicted herein without them.

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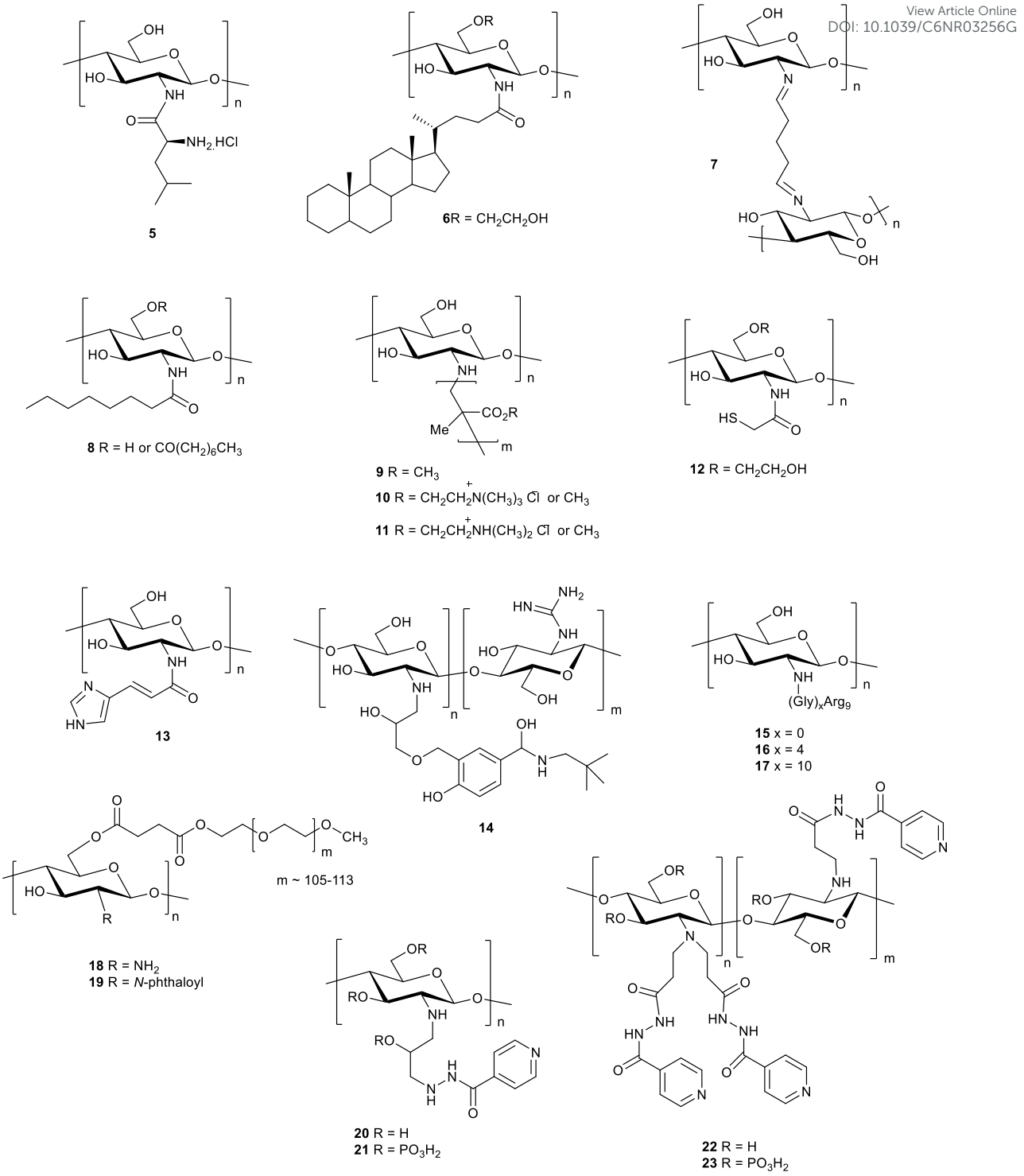

Fig. 2 Chemical structures of synthetic chitosan polymers investigated for lung drug delivery. For simplicity, the structures do not depict the presence of unmodified amino groups or *N*-acetyl groups which is some cases make up the majority of the monosaccharide units in the polymer, depending on the degree of substitution.

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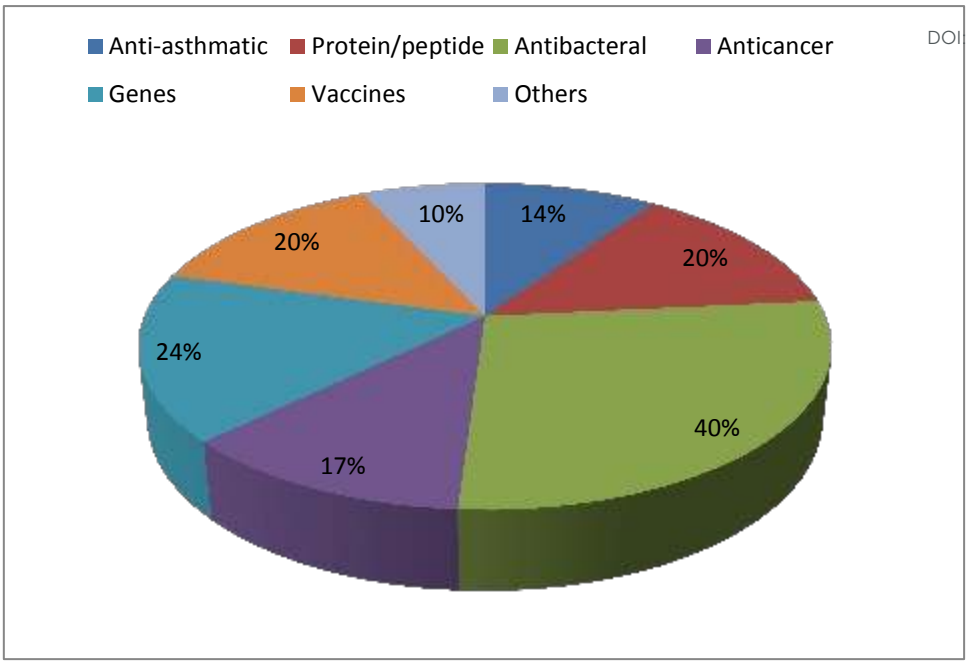

Fig. 3 Chitosan based lung delivery of various therapeutic agents investigated

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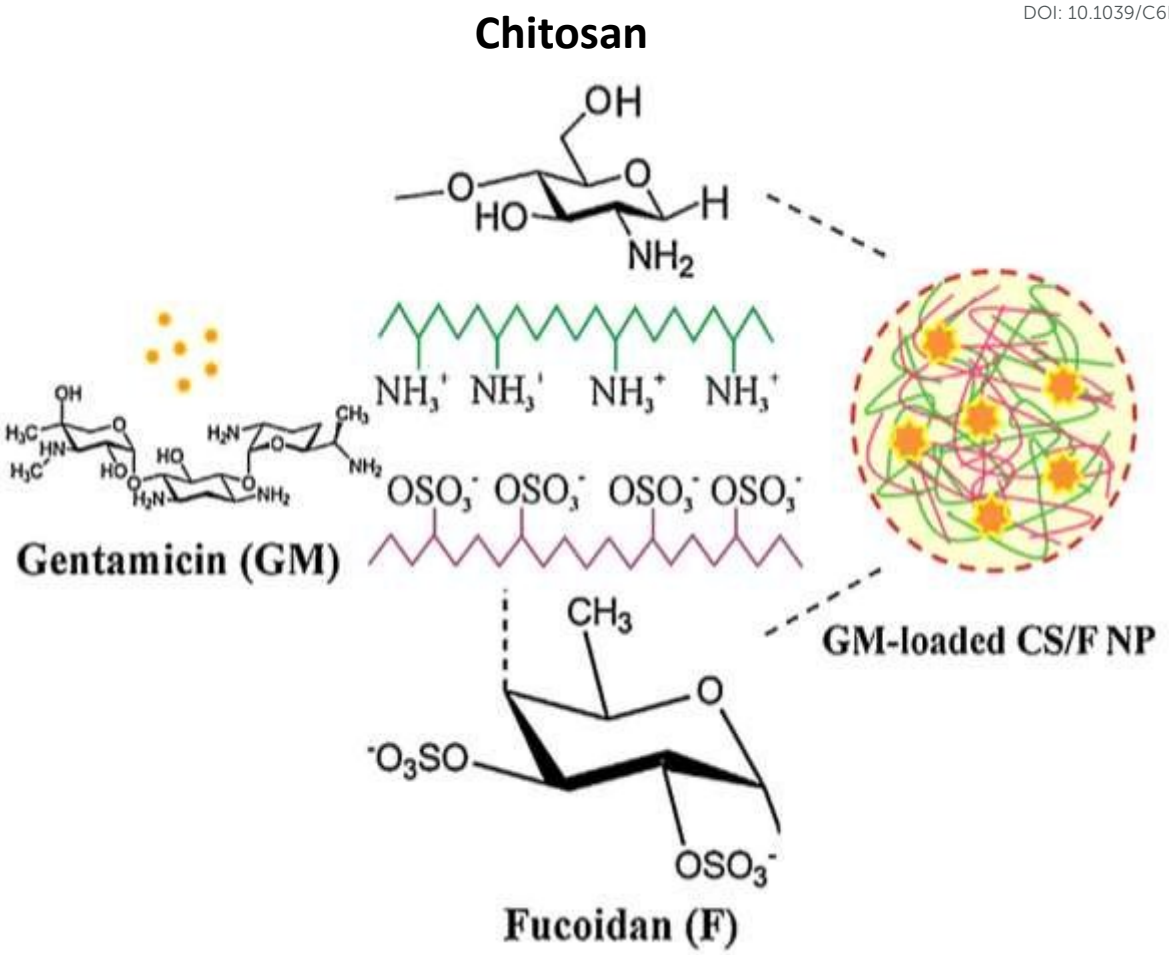

Fig. 4 Schematic illustration of the preparation of gentamicin loaded-CS/F NPs prepared by an ionotropic cross-linking method (Reproduced with permission).[78]

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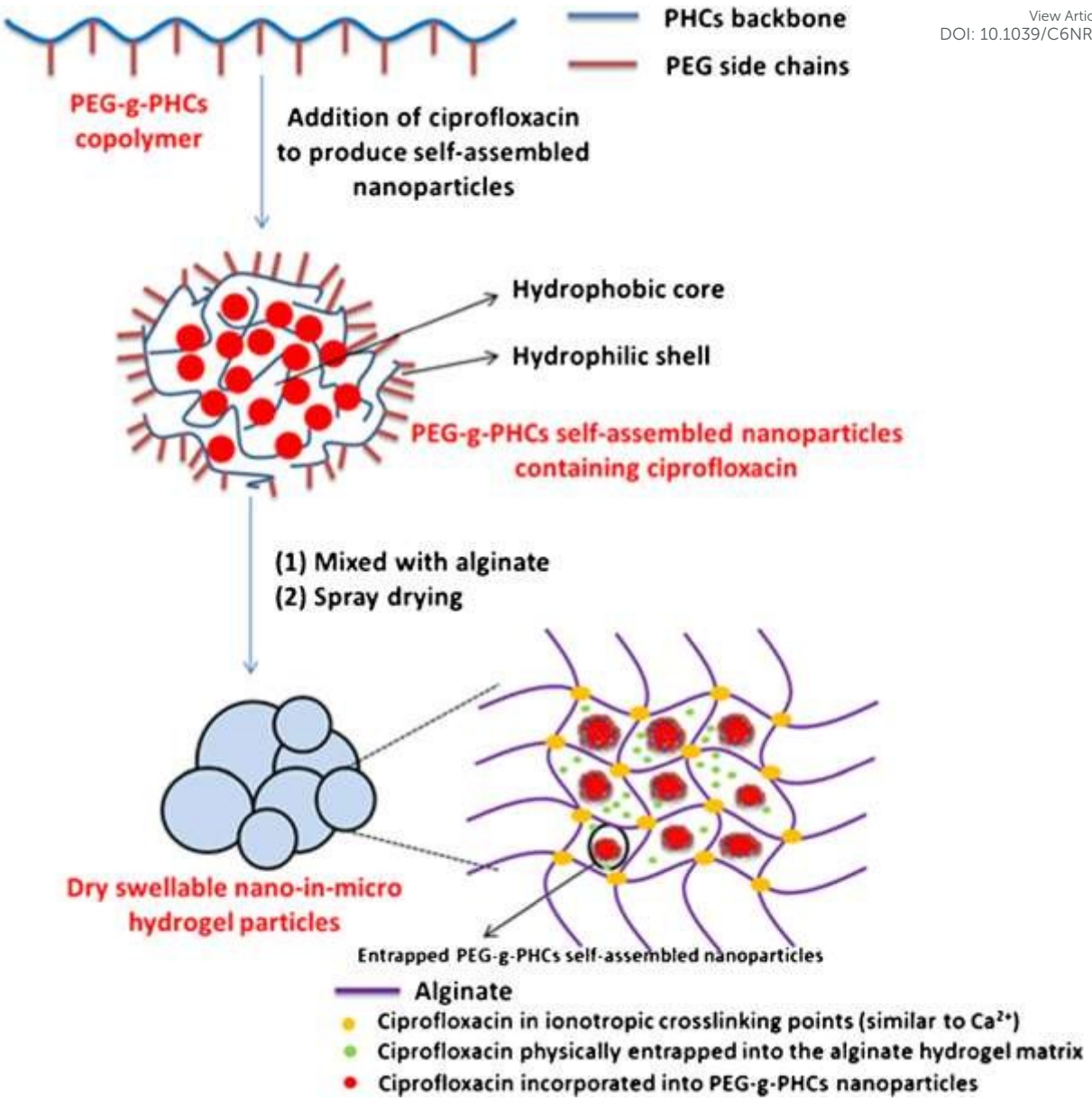

Fig.5 Illustration for the preparation of dry, swellable, nano-in-micro hydrogel particles using PEG grafted CS (Reproduced with permission).[108]

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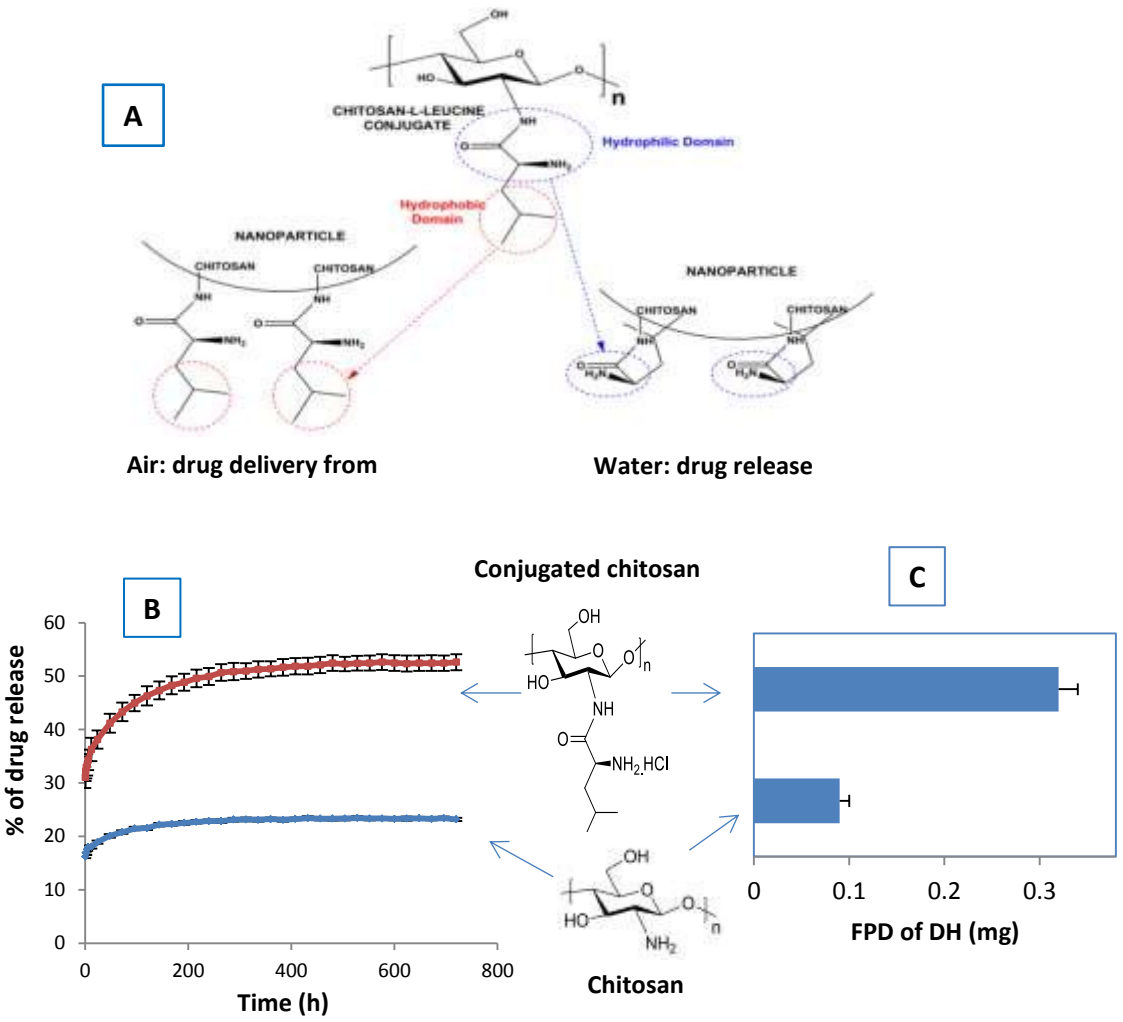

Fig.6. A. Leucine-conjugated CS nanoparticles showing hydrophilic and hydrophobic domains and the hypothesised nanoparticle surface with a conformation that changes to be hydrophobic in air (for dispersion from DPI formulation) and hydrophilic in water (for drug release); B. In vitro rapid and prolonged release profiles of drug from conjugated chitosan NPs; C. Enhanced drug deposition (fine particle dose FPD) from conjugated CS NPs (Reproduced with permission).[11]

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