O- palmitoyl chitosan-PEG micelles for amphotericin B delivery to the lung

Chavan T[1], Taylor KMG[1], Saleem IY[2], Randle LE[2], Elsaid Z[1], Somavarapu S1

1. Department of Pharmaceutics, the School of Pharmacy, University of London, 29-39 Brunswick Square, London, WC1N 1AX, UK. 2. School of Pharmacy and Biomolecular Science, Liverpool John Moores University, Liverpool, UK.

Summary

Fungi such as Aspergillus and Canadida are ubiquitous in nature and can lead to severe lung infections. When treating these conditions, one must consider targeted drug delivery to the lung. Amphotericin B is the drug of choice for the treatment of fungal lung infections. Pulmonary delivery of antifungal agents is complicated as a result of their poor aqueous solubility and toxicity. Amphiphilic chitosan derivatives may be particularly appropriate for this application. The amphiphilic nature is achieved by the addition of the hydrophobic group: Palmitoyl and hydrophilic group: PEG, which will also enhance the stability and hydrophobic nature of polymer. These carriers can form micelles or complexes which are in the size range 40-50 nm + 7.89 nm with a charge of +9-11 mv + 1.55 mV. Grafting was confirmed by FT-IR and 1H NMR. Thermodynamic properties were also determined by Differential Scanning Calorimetry, which showed a glass transition temperature of 135°C. Transmission Electron Microscopy showed small and uniform spherical micelles/complexes were produced. UV spectroscopy was used to assess the drug loading efficiency within the carriers, showing a value of 30-40% with 1:10 ratio of the drug:polymer and 50-60 % with 1:5 ratio of the drug:polymer. Aerosol deposition properties were assessed using a Twin Stage Impinger following delivery from a iet nebuliser, Which gave deposition of 65.28 + 1.1 % in stage 2. No toxic effects of amphiphilic chitosan derivative were observed in bronchial epithelial cultured cell lines up to a range of 500mcg/ml

Introduction

Aspergillus and Candida species are transmitted via inhalation and may cause colonisation of the airways (alveolar macrophages) leading to life threatening diseases, especially in immunosuppressed patients (Vyas et al., 2005; Gilani et al., 2011) . It is very difficult to treat lung fungal infections as, systemic delivery does not efficiently deliver drugs to the site of infection, and most of the antifungal agents are toxic. Amphotericin B is the drug of choice for systemic fungal infections but produces nephrotoxicity which is dose limiting and can cause permanent harm (Albasarah et al., 2010). To reduce toxicity associated with amphotericin B, it should be targeted to the site of infection so that there will be no interaction of drug with non-targeted tissues. However it has very poor solubility in many organic solvents and is completely insoluble in water, this combined with toxicity necessitates the use of delivery system for delivery of amphotericin B to the lung (Vyas et al., 2005). In the literature Chitosan based liposomes and micelles have been used for the delivery of amphotericin B by nebulisation (Albasarah et al.,2010; Gilani et al., 2011).

Chitosan is an aminopolysaccharide obtained by partial alkaline deacetylation of chitin from a number of invertebrates (crustaceans' exoskeleton, insects' cuticles etc) and cell walls of fungi and others. (Joshi and Sinha, 2006; Inmaculada et al.2010). Chitosan is a hydrophilic polymer, offering attractive properties like biocompatibility, biodegradability, low-toxicity, non-immunogenicity, non-carcinogenicity, stability, abundance, antifungal and antimicrobial properties (Tong et al., 2005; Joshi et al., 2006; Huang et al., 2010). Insolubility in organic solvents and water are major disadvantages of chitosan. To overcome this, in this study a hydrophobic group such as palmitoyl was added at the O-position and then PEG was conjugated. PEG imparts increased stability and additional hydrophilic nature to chitosan. Hydrophobic group can be attached at two positions, either at O-Acyl or at N-Acyl position, but the addition of a hydrophobic group does not improve solubility in chlorinated solvent, such as chloroform and dichloromethane. This solubility is improved with grafting of the hydrophobic group at the O-Acyl position. The overall carrier is amphiphilic, forming micelles which are small in size. Micelles form a hydrophobic core and hydrophilic shell hence providing a good carrier system for the delivery of hydrophobic drugs, with the latter being encapsulated in the hydrophobic core. Polymeric micelles are mechanically more stable (Zhang et al., 2007). The polycationic nature of chitosan potentially also increases delivery of the drug to the negatively charged lung airway.

Pulmonary drug delivery utilises nebulisers, dry powder inhalers (DPI) and pressurized metered dose inhalers (pMDI) for the delivery of drug components either in the form of a solution/suspension or dry powder. With a nebuliser drug can be inhaled during normal breathing through a mouth piece or face mask, increasing its suitability for elderly patients and children. In additions, nebulisers also deliver relatively large

volumes of solution/suspensions and are therefore suitable for drugs with larger therapeutic doses (McCallion et al., 1996). The aim of this study was to synthesis and characterise O- palmitoyl chitosan-PEG carriers for the delivery of amphotericin B to the lung using jet nebuliser.

Materials:

Chitosan [molecular weight (MV) = 3-5K] was purchased from Kitto Life Co. Ltd (South Korea). Amphotericin B, methane sulfonic acid, palmitoyl chloride, sodium bicarbonate and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich Co. Ltd., UK. Methanol, dichloromethane and chloroform purchased from Fisher Co. Ltd., UK. mPEG NHS was purchased from Advanced Polymer Materials Inc., Canada.

Methods:

Synthesis of O- palmitoyl chitosan-PEG

O- palmitoyl chitosan was synthesized according to procedure described by Huang et al 2010, with some modification. Chitosan was dissolved in methane sulfonic acid and palmitoyl chloride was added to this solution. The reaction was stopped by the addition of ice. The solution was dialyzed against water and treated with sodium bicarbonate and again dialyzed against water. The solution was then lyophilized to produce O- palmitoyl chitosan. Thereafter, the O- palmitoyl chitosan produced was conjugated with PEG by dissolving mPEG NHS and O- palmitoyl chitosan in anhydrous dichloromethane. The product of this reaction was dialyzed against water. The solution was lyophilized to get novel O- palmitoyl chitosan-PEG.

Producing Nanocarriers:

The thin film method was used to produce nanocarriers with O- palmitoyl chitosan-PEG and amphotericin B, where chloroform and methanol were used as solvents and the film was hydrated with water at 60°C.

Characterisation:

The polymers produced were then characterized for solubility, grafting by FT-IR and ^{1}H NMR, size and zeta potential, morphology was seen with Transmission Electron Microscopy by dispersing 2 mg of polymer in water and the thermal properties were determined by Differential Scanning calorimetry (DSC). *In vitro* testing of polymer for MTT assay to determine cell viability on exposure to O- Palmitoyl Chitosan-PEG was performed using a bronchial epithelial (16HBE 14o-) cell line, seeding was done in 96 well plates and treated with increasing concentration of O- palmitoyl Chitosan (0-100 mcg/ml) for 24 h and absorbance was measured using spectroscopy at 570 nm and was corrected for background absorbance.

Nanocarriers were characterized for size and zeta potential, surface morphology by Transmission Electron Microscopy, drug loading by UV and aerosol deposition characteristics were determined following delivery from a jet nebuliser (Pari LC Sprint) into a Twin Stage Impinger (Copley Instruments, UK) operated at 60L/min with water as collection medium in the two stages.

Results and Discussion:

O- palmitoyl chitosan showed good solubility in water and organic solvents like chloroform, DCM and DMSO. Polymer was dissolved in water and characterized by a Malvern Zeta sizer Nanozs (Malvern Instruments Ltd., UK) for size and zeta potential. Mean size was in the range of 45 + 7.89 nm. Zeta potential was positive and in the range of 13.5 + 4.61 mV. Morphology was observed using a Philips CM 120 BioTwin transmission electron microscope (Philips Electron Optics BV, The Netherlands) and showed uniform, spherical micelles of a smaller size as shown in Fig. 1 (a). FT-IR of chitosan and its derivatives was undertaken using a Perkin Elmer FT-IR spectrometer, as shown in Fig 4. FT-IR of chitosan and its derivatives showed chitosan peaks at 1150, 1059, 1026 and 893 cm-1, palmitoyl peaks at 2917, 2850 and 1737 cm-1 and PEG groups are seen at 2852, 951 and 845 cm-1. Additional information of acyl chitosan was obtained by ^{1}H NMR (CDCl3), shown in Fig. 2, which showed peaks for the palmitoyl group at 0.90 (t, 3H), 1.10-1.45 (m 27.6H), 1.61 (s, 2.6H), 1.8-2.1 (br, 0.3H), 2.35 (m, 2.6H), 2.7-2.9 (br, 0.5H) and 3.2-5.2 (m, 4.3H) ppm. DSC showed a Tg value at 135°C indicating the amorphous nature of polymer.

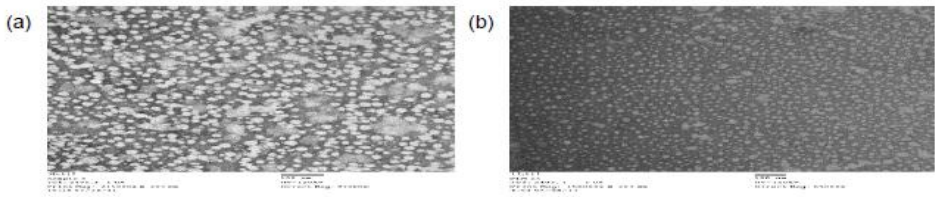

Fig. 1: TEM image a) O- palmitoyl Chitosan-PEG micelles b) Amphotericin B loaded micelles

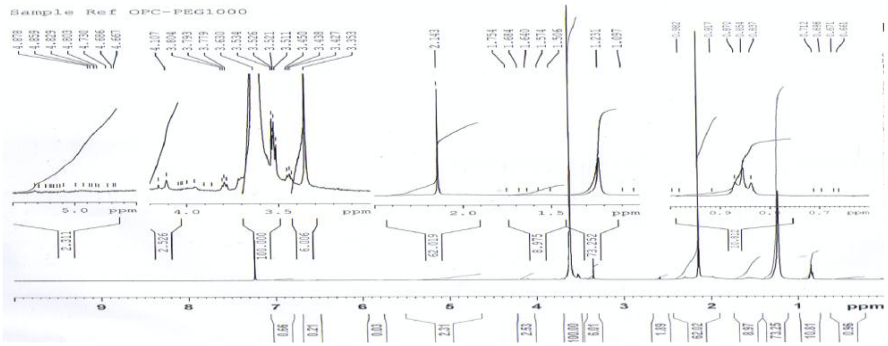

Fig. 2: ^{1}H NMR of O-palmitoyl chitosan-PEG

Exposure of the cell line to increasing concentrations (62.5 – 500 mcg/ml) of O-palmitoyl chitosan -PEG, resulted in equivalent cell viability to control (Fig. 3), with 81.82 ± 16.08 % of cell viability at 500 mcg/ml. However, increasing the dose to 1000 mcg/ml resulted in cell viability at 53.74 % ± 12.96. Therefore formulations are relatively non-toxic up to 500 mcg/ml.

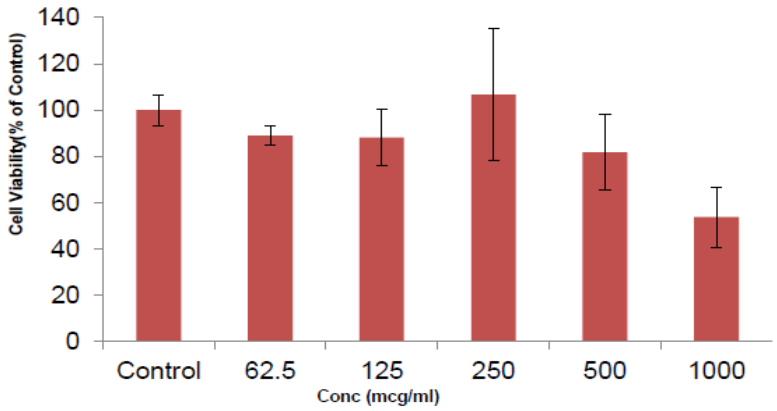

Fig. 3: Concentration Vs Cell viability of O- palmitoyl chitosan-PEG

Nanocarriers containing amphotericin B were characterized for size and zeta potential using a Malvern Zeta sizer Nanozs (Malvern Instruments Ltd., UK), giving a mean size of 45 + 7.89 nm and zeta potential of +10 + 1.55 mV. Although the size of these carriers was seen to be the same as that of the polymer without drug, there was a slight reduction in the zeta potential. Drug loading efficiency was assessed using a Perkin Elmer UV lamda 25 spectrometer. According to the ratio of drug:polymer used, the drug loading was seen to range from 25-40 % and 40-60 %, with the higher drug loading being achieved in the formulation with a lower ratio of drug to polymer.

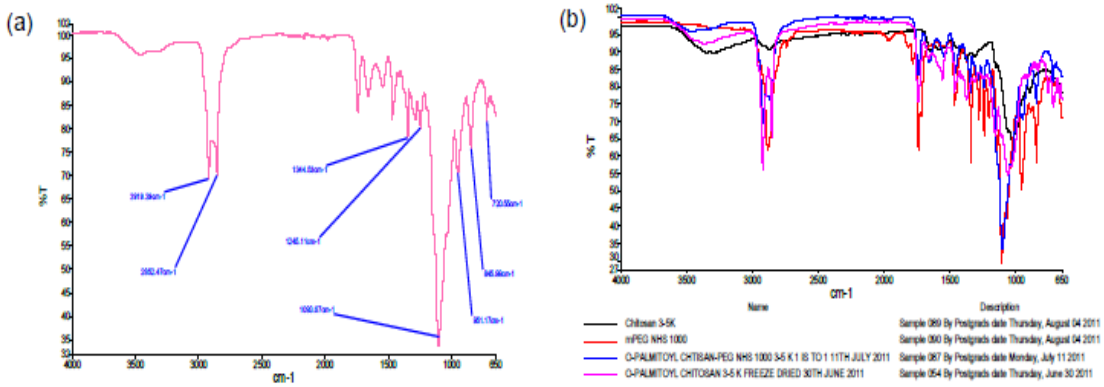

Fig. 4: FT-IR of O, Palmitoyl Chitosan-PEG: a) O, almitoyl Chitosan-PEG, b) Comparison of Chitosan, O Palmitoyl Chitosan-PEG, mPEG NHS and O, Palmitoyl Chitosan-PEG.

On aerosolisation of the polymer with drug, using a jet nebuliser, 65% of the drug was deposited in to the lower stages of the impinger as fine particle fraction

Conclusions:

Positively charged, small, spherical micelles which were uniform in size distribution were produced with O-palmitoyl chitosan-PEG, with up to 50% of drug loading efficiency. Positively charged micelles can efficiently deliver the negatively charged antifungal agent amphotericin B to the negatively charged lung, hence offering a potential carrier for pulmonary delivery of amphotericin B. *In vitro* cell culture studies showed non toxicity of formulation at concentrations up to 500 mcg/ml.

References:

1. Albasarah,Y. Y., Somavarapu, S., Stapleton, P. & Taylor, K. M. G. 2010. Chitosan-coated antifungal formulations for nebulisation. *Journal of Pharmacy and Pharmacology,* 62, 821-828.
2. Gilani, K., Moazeni, E., Ramezanli, T., Amini, M., Fazeli, M. R. & Jamalifar, H. 2011. Development of respirable nanomicelle carriers for delivery of amphotericin b by jet nebulization. *Journal of Pharmaceutical Sciences,* 100, 252-259.
3. Huang, Y., Yu, H., Guo, l. & Huang, Q. 2010. Structure and self-assembly properties of a new chitosan-based amphiphile. *The Journal of Physical Chemistry* b, 114, 7719-7726.
4. Inmaculada, A., Ruth, H., Angeles H. 2010 Chitosan amphiphilic derivatives. Chemistry and applications. *Current organic Chemistry* 14, 308-330.
5. Mccallion, O. N. M., Taylor, K. M. G., Bridges, P. A., Thomas, M. & Taylor, A. J. 1996. Jet nebulisers for pulmonary drug delivery. *International Journal of Pharmaceutics*, 130, 1-11.
6. Tong, y., Wang, S., Xu, J., Chua, B., He, C. 2005. Synthesis of O, O'-diPalmitoyl chitosan and its amphiphilic properties and capacity of cholesterol absorption. *Carbohydrate Polymers*, 60, 229-233.
7. Vyas, S. P., Quraishi, S., Gupta, S. & Jaganathan, K. S. 2005. Aerosolized liposome-based delivery of amphotericin b to alveolar macrophages. *International Journal of Pharmaceutics*, 296, 12-25.
8. Zhang, C., Ding, Y., Yu, l. & Ping, Q. 2007. Polymeric micelle systems of hydroxycamptothecin based on amphiphilic n-alkyl-n-trimethyl chitosan derivatives. *Colloids and Surfaces B: Biointerfaces*, 55, 192-199.