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Synthesis and characterization of self-assembling chitosan-based nanoparticles

Junaid Qureshi[1], Furqan Muhamad Iqbal[1*], Zeeshan Danish[2], Syed Nisar Hussain Shah[1], Hasaan Umar[3], Muhammad Zaman[4], Muhammad Nadeem Alvi[4] and Nayyer Islam[5]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan
[2]University College of Pharmacy, University of The Punjab, Lahore, Pakistan
[3]Department of Pharmacy, Hajvery University, Lahore, Pakistan
[4]Faculty of Pharmacy, University of Central Punjab, Lahore, Pakistan
[5]Department of Pharmacy, University of Lahore, Gujrat Campus, Pakistan

Abstract: Chitosan (CHT) based biodegradable nanovectors were synthesized and modified with poly ethylene glycol 4000 (PEG-4000). CHT having medium molecular weight with 75% to 85% deacetylation was phthaloylated with phthalic anhydride, followed by PEGylation using PEG-4000. After confirmation of successful PEGylation by fourier transforminfra red spectroscopy (FTIR), the modified polymer was further processed to develop the nanocarrier using ionic gelation method by the addition of sodium tripolyphosphate (NaTPP). The prepared nanocarriers were subjected to physicochemical evaluation. The surface morphology of the particles was observed under scanning electron microscope (SEM), and particle size by dynamic light scattering (DLS) method, which was about 159-170nm in diameter. The zeta potential of the prepared nanovectors was +0.907mV which was due to cationic nature of nanovectors. The cell viability studies were also conducted to find the suitability of the carrier for *in-vivo* application, using liver cancerous cells (Hep G2). The findings have disclosed the concentration dependent activities of the particles, as viability of the cell was shown to be decreased with the increase in the concentration of the particles. Conclusively, the study was successful in determining the toxicity profile of these nanovectors as these were proved non-toxic at specific concentration.

Keywords: Chitosan, poly ethylene glycol, sodium tripolyphosphate, nanocarriers.

INTRODUCTION

Nanotechnology is the maneuvering of atoms and molecules with dimensions and size range of up to 100nm. It is a technique which enables to handle and manipulate the ranges of matters in nanometers. So, as a whole, many studies are being conducted on nano-materials to utilize their properties and most importantly, to use them as nanovectors for transporting targeted moiety (Chellaram *et al*., 2014; Nguyen *et al*., 2017).

CHT is a naturally occurring polysaccharide comprised of alkaline N-deacetylated of chitin. It is soluble in aqueous acidic solvent and is known as chitosan. Due to its aqueous-acidic medium soluble property, it has the ability to form gels, fibers and films (Rinaudo, 2006). Chitosan is a nontoxic, biodegradable, biocompatible and semi-crystalline polysaccharide and is not abundant in nature but is extracted from abundant chitin by deacetylation process. Chitosan has many biological properties, which make it useful in pharmaceutical and tissue engineering. Chitosan has mucoadhesive property and it binds to cell and offers greater time for drug to penetrate to the cell. CHT has a positive charge, which makes it ideal for drug delivery systems (Riva *et al*., 2011). Its molecular weight controls the complex formation with DNA or RNA, as it has been observed that high MW of chitosan forms better complex, but on the other hand, it hinders the dissociation in cell (Mao *et al.,* 2010) (Villegas *et al*., 2021).

Ionic gelation is specifically used when CHT gelation is induced by small anionic molecules like citrate, sulfate, phosphate etc. This simple process requires ionic interaction between the cationic amino groups of CHT and NaTTP (Carvalho *et al*., 2009; Nishimoto *et al*., 2021). The objective of the study was to synthesize PEGylated chitosan-based nanoparticles (PEG/CHT-NPs) for biomedical applications. In future, the current study could influence the application of PEG/CHT-NPs based targeted delivery.

MATERIALS AND METHODS

Medium-molecular-weight CHT with 75% to 85% deacetylation ranges and viscosity of 200cps to 800cps was obtained from Sigma-Aldrich, Germany. PEG-4000, anhydrous N, N-Dimethyl formamide (DMF), anhydrous tetra hydrofuran (THF), agarose powder, potassium dihydrogenphosphate, glacial acetic acid, hydrazine monohydrate, methyl alcohol, potassium chloride, pyridine, sodium tripolyphosphate (TPP), sodium hydride (NaH) and thionyl chloride (SoCl2) were purchased from Merck Germany. For cell viability assay, DMEM, 10%

*Corresponding author: e-mail: furqaniqbal@bzu.edu.pk

fetal bovine serum and MTT-reagent were also obtained from Sigma-Aldrich Germany.

Method

Preparation of chlorinated CHT

Accurately weighed 5g of medium molecular weight CHT was added to 40% w/v of aqueous sodium hydroxide (NaOH) solution. Then the solution was stirred for 4 hours under nitrogen environment, and at 110ºC. The resultant solution was vacuum filtered and treated with 40% w/v NaOH. The product obtained was freeze-dried for the next step of phthaloylation (Nishimoto *et al*., 2021).

Preparation of phthalated CHT

Solution of phthalic anhydride solution (13.8%) in dimethyl formamide (DMF) was used to mix 1.00 g of deacetylated CHT. The following solution was then stirred at 120ºC under nitrogen atmosphere for 8 hours, and then cooled down to room temperature, followed by precipitation in ice cold water. The precipitates were filtered and washed with methanol thoroughly and then vacuum dried (Karuna *et al*., 2020).

Synthesis of PEGylated CHT

The synthesis of PEGylated chitosan comprised of three steps; first, was the chlorination of CHT using Oxalyl chloride, second; entailed the activation of PEG using sodium hydride and the third included embedding of PEG to the chlorinated CHT solution. For the preparation of chlorinated CHT, 100mg of phthaloylated CHT was dissolved in 20mL of pyridine and then 10-fold of thionyl/Oxalyl chloride was added against the 100mg of phthaloylated CHT. The solution was stirred for 30 minutes at 80ºC under nitrogen environment, and then cooled at room temperature. The solution was then precipitated in ice cold water and vacuum dried. The activation of PEG was achieved by adding 4g of PEG-4000 to suspension of sodium hydride (NaH) in 50mL of anhydrous tetrahydrofuran. This suspension was stirred for 2 hours at 60˚C under nitrogen atmosphere. Then after 2 hours, the chlorinated-phthaloyl CHT weighing 60mg was added to the reaction solution and stirred for another 16 hours. The resultant solution was cooled at room temperature and precipitated in methanol. Finally, it was filtered and then vacuum dried (Yin *et al*., 2020). Then, 100mg PEGylated-phthaloyl CHT was taken and added in 15mL of hydrazine monohydrate and 30mL of distilled water was added to the mixture, which was then heated for 16 hours at 100˚C under constant stirring. The excess hydrazine monohydrate was removed by rotary evaporator. The process was repeated three times by diluting the mixture with distilled water and evaporating each time until a solid residue was obtained and vacuum dried (Malhotra *et al*., 2011).

Formulation of PEG/CHT-NPs

Ionic gelation process was used in order to obtain PEG/CHT-NPs. In this process, PEGylated CHT was added to 1% (v/v) of acetic acid solution by making a concentration of 0.5mg/mL and the pH of the solution was adjusted to 5. Solution 2 was prepared by dissolving NaTPP in double distilled water to obtain a concentration of 0.7mg/mL and pH of solution was adjusted to 3. Then, the solution 2 was added drop-wise to the solution 1 and stirred constantly at room temperature for 1 hour (Yan *et al*., 2020).

Confirmation of PEG/CHT conjugation

Fourier Transform-Infrared Spectroscopy was used in order to evaluate the chemical conjugation of PEG-4000 and CHT. The spectrum was drawn in the range of 500 to 4000cm^{-1} (Ismik *et al*., 2020).

Zeta potential of PEG/CHT-NPs

Zeta potential is the potential difference between the surface of the particle and the bulk liquid in which it is immersed. Stability of the system depends upon the zeta potential, as strong anionic and strong cationic systems are more stable than systems with low zeta potential (Melo *et al*., 2021).

Surface Morphology of PEG/CHT-NPs

Scanning electron microscope (SEM) was used in order to examine the surface morphology of the synthesized conjugate (Melo *et al*., 2021). The sample was mounted on the stub with double side adhesive tape, which was previously lyophilized and scattered.

Particle size analysis of PEG/CHT-NPs

The average particle size and its distribution were measured by dynamic light scattering using wavelength of 633nm and refractive index of 1.33 (Dogan *et al*., 2020).

Cytotoxicity Assay

The cytotoxicity assay for PEG/CHT-NPs was performed using liver cancerous cells (HepG2) and DMEM as a cell media. The cells were incubated and then incorporated with nanovectors samples. Finally, absorbance of the cells was measured at 500-600nm in ELISA reader.

STATISTICAL ANALYSIS

The data obtained during cytotoxic assay was analyzed by using GraphPad Prism version 7.

RESULTS

Confirmation of PEG/CHT conjugation

The chemical changes and conversion of CHT into PEG/CHT were evaluated by FTIR, that indicated the changes in chemical structure during chemical reactions (Dogan *et al*., 2020). CHT possessed the acetyl group in

its structure, and the peak at 1665cm^{-1} confirmed its presence in the parent molecule of CHT (fig. 1). After deacetylation, the peak disappeared with the emergence of amino group (N-H) at 1665cm^{-1}, along with stretching of bond at 3200-3500cm^{-1} wavelength (B). The deactylated CHT was subjected to the process of phthaloylation. The FTIR spectrum of phthaloylated CHT revealed sharp peaks at 1700 and 1775 cm^{-1} due to the presence of imide group (C). The changes in the spectrum of chlorinated CHT revealed elimination of peak at 3000cm^{-1} to 3400cm^{-1} due to the replacement of -OH group with chlorine (D). The appearance of peak at 2870cm^{-1} indicated the grafting of PEG/CHT (E). The disappearance of peaks at 1700cm-1 and 1775cm-1 indicated the dissociation of phthalimido group and the presence of peak at 1680cm-1 indicated the primary amine group in CHT (F).

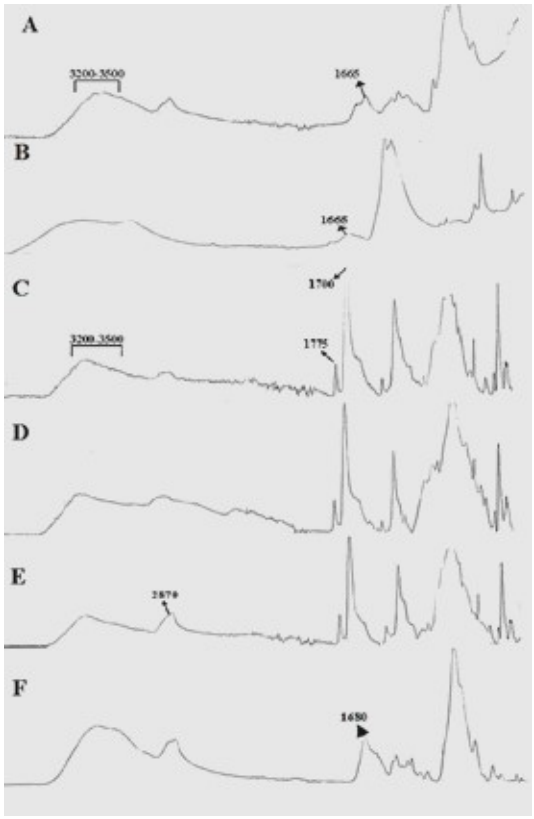

Fig. 1: (A) FTIR of medium molecular weight CHT, (B), FTIR of deacetylated CHT, (C) FTIR of phthaloylated CHT, (D) FTIR of chlorinated CHT, (E) FTIR of pegylated CHT and (F) FTIR of deprotacted CHT

Zeta potential of PEG/CHT-NPs

Zeta potential of PEG/CHT-NPs was found to be cationic having value +0.907Mv, which might be due to the cationic nature of the CHT.

Surface Morphology of PEG/CHT-NPs

Particle size analysis of PEG/CHT-NPs

The average particle size was measured by dynamic light scattering (DLS) technique and particle size distribution was evaluated by plotting a graph between intensity % and particle size in nanometer. The average particle size was found to be up to 170nm.

DISCUSSION

The deacetylation of chitosan was achieved by adding 40%w/v of aqueous sodium hydroxide (NaOH) solution which can be seen in fig. 2 of FTIR of deacetylated CHT. The peak at 1665cm-1 (>C=O) bent due to the presence of amine group (Czechowska-Biskup, Jarosińska, Rokita, Ulański, & Rosiak, 2012). Phthaloylation of CHT was achieved by stirring with DMF solution and fig. 3 indicates the presence two sharp peaks at 1700cm-1 and at 1775cm-1.

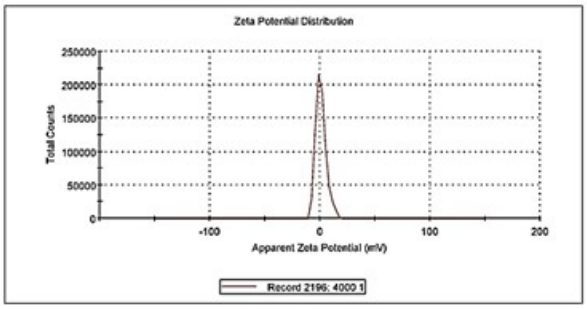

Fig. 2: Zeta potential of PEG/CHT-NPs

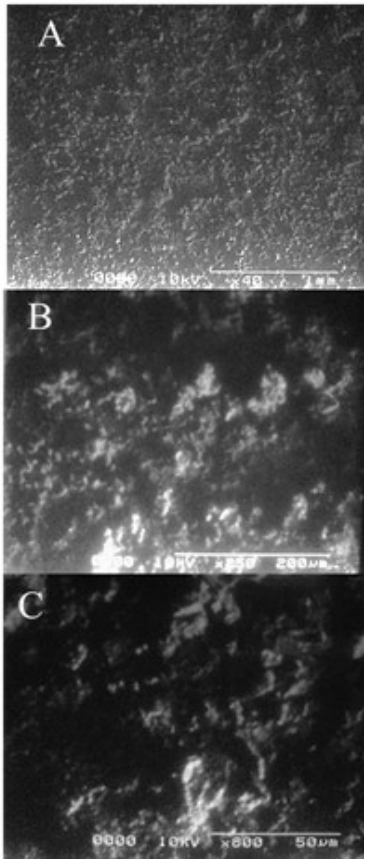

Fig. 3: SEM images of PEG/CHT-NPs at different resolution power, describing nanosized carriers of CHT.

These peaks indicate the presence of imides of phthaloyl group (A. Malhotra, Melville, & Watson, 2013). Phthaloylated CHT was further treated with thionyl chloride to achieve chlorination with the disappearance of peak at 3000cm-1 to 3400cm-1 in fig. 4 due to the replacement of -OH group with chlorine. The appearance of peak at 2870cm-1 in fig. 5 indicates the grafting of PEG-4000. Hydrazine monohydrate was added to pegylated CHT in order to deprotect it and fig. 6 reveals the elimination of peaks at 1700cm-1 and at 1775cm-1 due to complete dissociation of phthalimido group and the presence of primary and secondary amine group (Malhotra, Lane, Tomaro-Duchesneau, Saha, & Prakash, 2011).

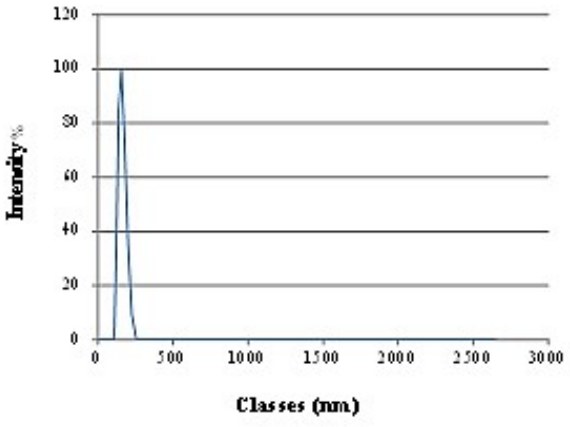

Fig. 4: DLS graph of particle size

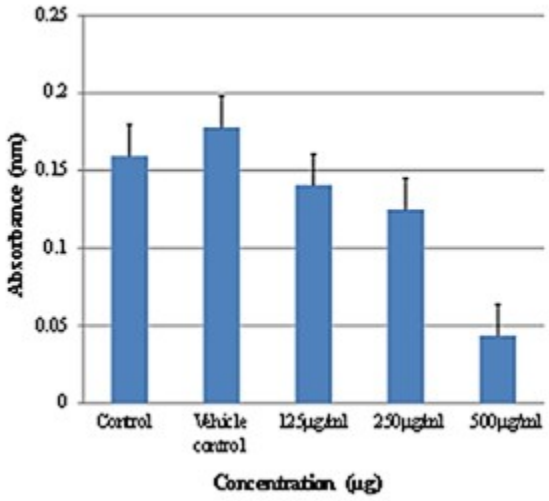

Fig. 5: Absorbance graph at different concentrations of PEG/CHT-NPs

The sample tested with dynamic light scattering contained the nanoparticles of medium molecular weight CHT modified with PEG 4000. The average particle size obtained had a diameter between 159-170 nm and can be authenticated by the literature (Ankit Jain, Kanika Thakur, Gajanand Sharma, Preeti Kush, & Upendra K Jain, 2016). While the polydispersity index (PDI) of the sample was 0.453, which indicates it as a mono-disperse sample with value in accordance with the literature studies (De Campos, Sánchez, & Alonso, 2001)(de Moraes *et al*., 2018). Zeta potential of the sample is +0.907mV which is slightly positive and is mainly due to the degree of deacetylation of CHT. By increasing the degree of deacetylation, the positive charge on the surface of nanoparticles also increases and from time to time, it decreases due to the instability of nanovectors. The designed nanovector indicated that it has a positive charge on its surface, which was desirable for the attachment of DNA, cell membranes and proteins, which are mainly anionic in nature. So, it appears to be an optimum nanovector with positively charged surface (Honary & Zahir, 2013). The surface morphology of the CHT nanoparticles was observed under scanning electron microscope (SEM). The sample of CHT nanoparticles was first lyophilized and then mounted on SEM. The images were taken at 1mm, 100µm and 50µm magnifications as shown in fig. 9. The surface of CHT nanoparticles seemed to be rough and dense due to the higher concentration of polymer (CHT) and also due to the cross linking interaction between CHT and sodium tripolyphosphate (TPP). The sample of lyophilized CHT nanoparticles was not coated with any metal so at higher potential, the sample got burned and image seemed blur (Romainor *et al.,* 2014). The cytotoxicity of CHT nanoparticles was performed using liver cancerous cells and MTT reagent as indicator. The control only contained the DMEM media and liver cancerous cells, the vehicle control contained DMEM media, liver cancerous cells and 1% acetic acid solution, which was used as diluent to dilute the nanoparticles concentration. Then different concentrations of CHT nanoparticles were used to determine cytotoxicity. The results showed that at low concentration of CHT nanoparticles, the cells survived and as the concentration increased, the absorbance decreased.

CONCLUSION

PEG/CHT has been achieved successfully, and processed to the development of nanocarriers. The prepared nanocarriers exhibited concentration-dependent cell killing abilities, indicating that they can not only be used as carrier but, may be considered as an effective anticancer approach.

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