Synthesis of Chitosan-Graft- HPβCD Copolymer by Novel One Pot Technique and its Application for Solubility Enhancement of Efavirenz

Aarti Belgamwar, Shagufta Khan, Lalit Rathi
Institute of Pharmaceutical Education and Research, Borgaon (Meghe), Wardha-442001, Maharashtra, INDIA

ABSTRACT
Objective: To enhance solubility of efavirenz by using chitosan-graft-HPβCD copolymer synthesized by novel one pot technique. Methodology: Poor aqueous solubility and limited bioavailability is the major problem for more than 40% NCE (new chemical entities). Efavirenz (EFV), widely used non-nucleoside reverse transcriptase inhibitor (NNRTI) belongs to BCS class II drug is having very poor intrinsic water solubility and limited oral bioavailability. Chitosan -graft-HPβCD (CS-g-HPβCD) copolymer was synthesized by tosylation of HPβCD followed by grafting on chitosan (CS) backbone. CS-g-HPβCD was prepared by varying CS: HPβCD weight ratios (1:1, 1:2, 1:3 and 1:4). The copolymer was characterized by FT-IR, NMR and DSC. The molecular weight of grafted copolymer was determined and copolymer was further evaluated for its solubility enhancement capability. Result: Grafted HPβCD copolymer was grafted on CS backbone by simple one pot synthesis method. FT-IR, NMR and DSC results avowed the synthesis of grafted copolymer. The molecular weight of grafted polymer was greater than molecular weight of CS also support confirmation of HPβCD grafting on CS. The copolymer was found to tremendously enhance aqueous solubility of EFV (380 times the solubility of drug in water). These results conclusively demonstrated synthesis of grafted copolymer possessing good potential in the solubility enhancement of the poorly water soluble drug like EFV.
Conclusion: Grafted copolymer is an excellent strategy to conquer the solubility issues of hydrophobic drugs like EFV owing to solubility enhancement capability and mucoadhesivity.

Key words: Chitosan, Chitosan-g-HPβCD, Efavirenz, Hydroxypropyl-β-cyclodextrin, Grafting, Solubility enhancement.

Correspondence:
Shagufta Khan,
Professor, Department of Pharmaceutics
Institute of Pharmaceutical Education and Research
Borgaon (Meghe) Wardha-442001, Maharashtra, INDIA.
Phone: 91 7152240284, Fax No. 91 7152241684
Email: shaguftakhan17@rediffmail.com
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INTRODUCTION
There has been growing interest in studies on graft co-polymerization of polysaccharide chitosan (CS) to introduce novel functions into this biopolymer. CS, a deacetylated derivative from chitin allows specific chemical modifications since it has primary amine group at the C-2 position and primary alcoholic group at the C-6 position of its monomeric units.1,2 Graft modification improves CS water solubility and bioactivities such as antibacterial, antitoxin,3 chelation,4 and complexation properties5 while maintaining its mucoadhesive and biocompatible properties.

Cyclodextrin (CD) grafted or cross linked with CS are now being developed into ‘smart’ systems for modifying drug release behavior, solubility and efficient targeted drug delivery especially for hydrophobic drugs. Cyclodextrin consists of (α-1,4)-linked α-D-glucopyranose units with lipophilic central cavity and hydrophilic outer surface. The hydroxyl functions are oriented to the cone exterior with the primary hydroxy groups at narrow edge (6th position) and the secondary at the wider edge (2th and 3th position). Recently, studies on the functionalization or modifications of CDs at the secondary side to alter its properties have gained interest.6,7,8

To date, various approaches have been proposed to link cyclodextrin to chitosan using different methods of preparation. As reported by Prabaharan and Mano,9 tosylation β-CD was linked to chitosan at the 2-position of CD. Chen and Wang,10 modified iodine release with chitosan-β-cyclodextrin-iodine complex. Lu Lu and co-workers11 conjugated β-CD to CS through click chemistry to synthesize copolymer chitosan-graft-β-cyclodextrin. They utilized chitosan-graft-β-cyclodextrin and carboxymethyl chitosan for formulation of Doxorubicin hydrochloride nanoparticles. Astaria Luzardo-Alvarez12 developed hydrophobically modified chitosan containing β-CD by tosylation which was used to obtain crosslinked microparticles. Poor solubility of CD-g-CS in water was improved by introduction of glycidyltrimethyl ammonium chloride as a quaternizing agent while this novel quaternized CD-g-CS (QCD-g-CS) showed higher antimicrobial activity.13 Inclusion complex of QCD-g-CS derivatives when studied with Eugenol expressed excellent mucoadhesion and higher antimicrobial activity.14 Present study is focused towards grafting of highly water soluble Hydroxypropyl-β-cyclodextrin (HPβCD) on CS backbone in order to obtain a copolymer having free water solubility, high yield and having capability to modify CS with hydrophobic cavities of HPβCD for better inclusion complex formation with chemical entities having low water solubility while maintaining mucosal adhesivity, permeability and biocompatibility properties of CS.

Efavirenz (EFV), first line non-nucleoside reverse transcriptase inhibitors (NNRTI) is used as a model drug. It belongs to BCS class II category having high lipophilicity (log P = 5.4), poor intrinsic water solubility (4µg/ml), limited oral bioavailability (40– 50%), with high intra and inter-individual variability of 19-24% and 55-58% respectively.15,16
In the present study, one-pot synthesis technique was used for grafting of HPβCD on CS backbone. The grafted copolymer was then studied for solubility enhancement of EFV.

**MATERIALS AND METHODS**

**Materials**

Chitosan was the generous gift from Mehtani Pvt. Ltd Gujrat, India. Hydroxypropyl-β-cyclodextrin was supplied by Roquettee India Pvt Ltd, Mumbai, India. Efavirenz was kindly supplied by Aurobindo Pharma Co. Hyderabad, India. All other chemicals used for study were of analytical grade.

**Methods**

**Preparation of Chitosan grafted hydroxy propyl-β-cyclodextrin (CS-g-HPβCD)**

Tosylated HP-β-CD (Ts-HPβCD) was synthesized with modifications of Brady et al.'s method. Briefly, HPβCD (1 g, mol wt 1391.221) was dissolved in 2 mL of distilled water containing NaOH (60 mg) at freezing temperature. P-toluene sulphonyl chloride (Ts-Cl) (1 g) was dissolved in Acetonitrile (3 mL) and added drop-wise to HP-β-CD solution with constant stirring for 6 hr by strictly maintaining freezing temperature. CS (mol. wt 435KDa) was grafted with HP-β-CD according to Goni’s method with modifications. Ts-HPβCD solution was mixed with CS solution in distilled water (1 g in 80 mL). Resultant solution was refluxed for 24 h at 50°C. It was dialyzed across the dialysis membrane-70 (Molecular weight cut-off 12,000 -14,000 kDa) in deionized water for 4 days. The solution was then freeze dried and cotton like fluffy powder was obtained. CS-g-HPβCD was prepared by varying CS: HPβCD weight ratios (1:1, 1:2, 1:3 and 1:4).

**Characterization of CS-g-HPβCD**

The grafted copolymer was characterised by FTIR, NMR, DSC and XRD for the confirmation of conjugation between CS and HPβCD and molecular weight was also determined.

**Fourier transform infrared spectra (FT-IR)**

The Fourier transform infrared (FT-IR) spectra of the of CS, HPβCD, physical mixture of CS with HPβCD (1:1), CS-g-HPβCD (1:1, 1:2, 1:3 and 1:4), EFV and physical mixture of CS-g-HPβCD with EFV were recorded on FTIR spectrophotometer (Shimadzu Asia Pacific Pvt. Ltd, Singapore spectrometer Model No – 84005) in the range of 4000-400 cm⁻¹ using KBr pellets.

**Nuclear magnetic resonance spectroscopy (NMR)**

The ¹H NMR spectra of CS and CS-g-HPβCD (1:1, 1:2, 1:3 and 1:4) were recorded on NMR (BRUKER BioSpin Group AVANCE II 400 MHz NMR spectrometer, Germany) using tetramethyl silane as an internal standard and D₂O as a solvent for dissolving at 25°C.

**Differential scanning calorimetry (DSC)**

Differential scanning calorimetric analysis was performed (Mettler Toledo AG, Analytical, Switzerland) to investigate the thermal and crystalline behavior of CS, HPβCD, physical mixture of CS with HPβCD (1:1), CS-g-HPβCD, EFV and physical mixture of CS-g-HPβCD with EFV (1:1). A heating rate of 10°C/min was employed in the range of 40–450°C. Analysis was performed under nitrogen purge (50 mL/min).

**Determination of molecular weight**

Molecular weight of CS-g-HPβCD was determined using Zeta sizer ZS90, (Malvern Instrument Ltd., UK) by static light scattering (SLS) process, a technique which characterises the molecules in the solution. Intensity of scattered light that a particle produces is proportional to the product of the weight-average molecular weight and the concentration of the particle. The sample at different concentrations were prepared in a range of 0.25 to 1 g/L using distilled water. Toluene was used as standard solvent. Absolute molecular weight was determined using Debye plot.

**Solubility determination**

Phase solubility study was carried out according to the Higuchi and Connors method. An excess amount of drug was added to 10 mL polymetric solution in distilled water. Solubility was determined with the ratios of CS and HPβCD (1:1, 1:2, 1:3, 1:4) in CS-g-HPβCD in increasing concentrations from 1mg/mL to 5 mg/mL. The series of suspensions were equilibrated by stirring at 25°C ± 0.5°C for 72 hr and then centrifuged. The supernatant was analyzed by double beam UV spectrophotometer (Model No-UV 2401 PC, Shimadzu, Japan) at 246 nm and amount of drug dissolved was calculated. Same procedure was repeated for pure drug in distilled water. The experiments were performed in triplicate, coefficient of variation associated with each measurement was never >3%.

**RESULTS AND DISCUSSION**

**Preparation of Chitosan grafted 2-hydroxy propyl-β-cyclodextrin (CS-g-HPβCD)**

Tosylation of cyclodextrin is a widely used method for the conjugation of primary hydroxy groups of the Tosylated-Cyclodextrin (Ts-CD) with other functional groups such as amino of CS. Tosylation is essential for the activation of –OH group of HPβCD. For grafting of Ts-HPβCD on CS backbone, a novel one pot synthesis technique was developed. Ts-HPβCD is obtained in solubilized form which directly reacted with CS under acidic environment at 50°C minimizing the reaction steps and time. The method resulted in the high yield of the copolymer (17 ± 2.64 %) and the copolymer had high water solubility.

One pot synthesis technique developed in the present study is simple, scalable and reproducible as compared to the previously reported methods such as method developed by Brady et al. where Ts-CD separates as a water insoluble precipitate which is to be dissolved in DMF before grafting on CS. While in the method reported by Goni et al., the solubility of CD-g-CS was improved by quaternization but it also increased the reaction steps.

The reaction pathway for the preparation of CS-g-HPβCD is given in following scheme 1.

**Figure 1:** Characterization of chitosan-g-CS-g-HPβCD copolymer.

**Characterization of CS-g-HPβCD**

**Fourier transform infrared spectra (FT-IR)**

The FTIR spectra of CS, HPβCD, physical mixture of CS with HPβCD, CS-g-HPβCD copolymer with varying ratios of CS to HPβCD (1:1, 1:2, 1:3 and 1:4), EFV and physical mixture of CS-g-HPβCD with EFV are depicted Figure 1A. The FTIR spectrum of pure HPβCD exhibited a broad band in between 3600 to 3100 cm⁻¹ due to O-H stretching and prominent peaks at 2929, 1645 and 1032 cm⁻¹ corresponds to C-H, H-O-H bending and C-O-C respectively. The FTIR spectrum of CS had...
a broad band in between 3600 to 3000 cm⁻¹ due to O-H and NH₂ groups whereas the absorption band at 1650 and 1379 cm⁻¹ corresponds to the C=O and C-O stretching of amide group and absorption band at 1606 due to NH₂ bending. CS showed absorption band at 1155, 1074 and 1029 cm⁻¹ due to the symmetric stretching of the C-O-C and involved skeletal vibration of the C-O stretching. In the FTIR spectrum of physical mixture of CS and HPβCD all the major absorption peaks CS and HPβCD were intact suggesting no interaction of polymers.

The FTIR spectra of copolymer prepared from CS:HPβCD in the ratios of 1:1 and 1:2 retained all the prominent bands of both the polymers suggesting failure of graft formation between the polymers while the FTIR spectra of copolymer prepared from CS: HPβCD in the ratio 1:3 displayed new bands which were comparatively more prominent in the copolymer formed from CS: HPβCD in the 1:4 ratio. There was disappearance of some of the main peaks of both the polymers along with major changes in the fingerprint region.

In the FTIR spectrum of EFV sharp peak at 3317 cm⁻¹ confirmed-NH stretching vibration which was supported by the evidence of N-H deformation at 1496 cm⁻¹. The absorption band at 1747 cm⁻¹ corresponds to C=O stretching vibration where as the absorption band at 2250 cm⁻¹ was due to −C=O. The spectrum showed the absorption band at 3093 and 1600 cm⁻¹ corresponding to the C-H stretching and −C=O-C stretching of the aromatic ring respectively. Absorption bands 690 and 652 cm⁻¹ corresponds to −CF stretch while bands at 1097 and 1074 cm⁻¹ corresponds to C-O-C stretch vibration. Absorption band at 1242 and 1166 cm⁻¹ confirms CN and CO groups respectively. All bands of EFV were found intact at their original frequency in the spectrum of physical mixture with copolymer suggesting no interaction of drug with co-polymer.

**Nuclear magnetic resonance spectroscopy (NMR)**

Figure 1B shows the ¹H NMR spectra of CS and its copolymer with HPβCD in the different ratios of CS: HPβCD (1:1, 1:2, 1:3 and 1:4) in D₂O at 400 MHz. The ¹H NMR spectrum of CS exhibited the singlet proton at δ4.73 ppm for −OH. The singlet protons at δ4.68 and δ4.48 ppm were assigned to H₁ and H₂ respectively where as the doublet at δ3.87 and δ3.71 ppm was assigned to H₃ and H₄ while the singlet at δ3.60 ppm was indicated for H₅ in CS. The singlet proton for H₆ was observed at δ2.86 ppm and singlet proton at δ1.97 ppm was assigned to −NH₂.

The ¹H NMR spectrum of copolymer prepared from CS and HPβCD in the ratio of 1:1 displayed the multiplet proton signals at δ3.93 – δ 3.41 ppm due to H₃, H₄ and H₅. The appearance of singlet proton at δ3.26 ppm was due to H₂ in the copolymer. The side chain protons of HPβCD appeared as a singlet signals at δ 2.93 and 2.77 ppm due to H₆, H₇ respectively. Doublet in the spectrum of copolymer at δ1.07 ppm was due to H₈ and singlet signal of proton at δ2.32 ppm was assigned to H₉. The ¹H NMR spectrum of copolymer exhibited new peaks, doublet of doublet proton signals, at δ7.6 – 7.2 ppm due to the aromatic protons of tosyl group. Doublet at higher side was assigned to ortho and lower side to meta protons. The singlet signals for H₁ and H₂ in copolymer were assigned at δ4.9 and δ4.7 ppm. The singlet proton signal at δ5.1 ppm was an indication of presence of −OH. There was no definite evidence for formation of copolymer between CS and HPβCD prepared with 1:1 ratio of CS: HPβCD. The ¹H NMR spectrum of CS and HPβCD copolymer in the ratio of 1:2, 1:3 and 1:4 exhibited the similar pattern of the signals with varying intensities. The spectrum did not reveal the evidence for the formation of copolymer even with 1:2 ratio however the NMR of copolymers of CS with HPβCD in the 1:3 and 1:4 ratios of CS: HPβCD displayed an additional strong band at δ 4.7 ppm revealing the formation of copolymer in between them. The ¹H NMR spectrum of copolymer in the ratio of 1:4 showed the involvement of −OH group of HPβCD with NH₂ group of CS in copolymer formation.
DSC thermograms of CS, HPβCD, physical mixture of CS and HPβCD (1:1), CS-g-HPβCD copolymer, EFV and physical mixture of CS-g-HPβCD with EFV (1:1) are shown in Figure 1C. CS exhibited a broad endothermic peak at 258.07°C and exothermic peak above 310°C due to melting followed by decomposition of CS backbone. DSC thermogram of HPβCD showed the initial loss of water up to 80°C followed by sharp endothermic peak at 109.71°C due to melting of HPβCD. In the physical mixture of CS and HPβCD endothermic peaks of both CS and HPβCD were retained at 310 and 109°C respectively which reflect compatibility between polymers. Endothermic peak of CS in CS-g-HPβCD thermogram was found to shift to lower temperature (231.46°C) as compared to pure CS. The shift in thermograms in copolymer was attributed to decrease in thermal stability as a consequence of decrease in crystalline nature. The shift in endotherms and exotherms of the copolymer to lower temperatures reveals early melting and decomposition of the copolymer. Substitution of HPβCD into the CS backbone had reduced the strength of the backbone.

EFV thermogram showed a sharp melting endothermic peak at 139.16°C and exothermic peak attributed to decomposition at 265°C. The compatibility of EFV with CS-g-HPβCD copolymer was confirmed in physical mixture as there was no change in thermal behavior of EFV.

Molecular weight determination

The weight-average molecular weight (Mw) was determined from Debye plot from the intercept at zero concentration shown in Figure 1D. The molecular weight of CS-g-HPβCD was found to be 747±25 KDa which was greater than molecular weight of CS (435 KDa). Increased molecular weight supports confirmation of grafting of HPβCD on CS backbone.

Solubility determination

Phase solubility curve was found to be A₁ type (Figure 2) as linear increase in drug concentration was observed as a function of grafted polymer. The slopes of the linear ($r^2 = 0.999$) regions are all less than one, suggesting the formation of 1:1 drug/CD complex. Equilibrium solubility of EFV in distilled water was found to be 0.0168 mg/mL. Solubility of EFV with grafted copolymer having 1:4 ratio of CS and HPβCD was found to increase solubility of EFV 380 times than that of the free drug. This increase in solubility is higher than the techniques reported earlier such as nanosuspension, solid ternary system containing EFV, MβCD and PVP K30, dendrimerization, composite hydrogel containing HEC-PAA and nanocrystallization.

It was observed that with the increase in ratio of HPβCD in graft copolymer (1:1, 1:2, 1:3, 1:4) solubility of EFV was also increased. This might be attributed to combined effect of increased substitution of HPβCD on CS. Also, copolymer offers more cup like structure through conjugation of HPβCD with CS. This improved solubility may be due to the formation of inclusion complex between the hydrophobic cavities of CS-g-HPβCD and EFV. This finding is corroborated by the DSC thermogram. Increase in the solubility of EFV may be due to the formation of hydrogen bond between N-H and 'O' atoms of drug with –OH group of polymers.

CONCLUSION

Novel water soluble CS-g-HPβCD grafted copolymer was successfully synthesized by tosylation using simple one-pot synthesis method. The solubility issues of EFV have limited its use in the treatment of AIDS. Grafted copolymer is an excellent strategy to conquer the solubility
issues of hydrophobic drugs like EFV owing to solubility enhancement capability and mucoadhesivity. Authors are optimistic that the novel CS-g-HPβCD will offer great opportunity as a carrier system with widespread application in developing novel drug delivery system for application across the transmucosal systems like nasal, ocular, vaginal and rectal.

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CONFLICT OF INTEREST
No conflict of interest are declared.

ABBREVIATION USED
CS: Chitosan; CD: Cycloextrin; QCD-g-CS: Quaternized CD-g-CS; HPβCD: Hydroxypropyl-β-cycloextrin; EFV: Efavirenz; NNRTI: Non nuclease reverse transcriptase inhibitor; CS-g- HPβCD: Chitosan-grafted-Hydroxypropyl-β-cycloextrin; TS- HPβCD: Tosylated-HPβCD; FTIR: Fourier transform infrared spectra; NMR: Nuclear magnetic resonance spectroscopy; DSC: Differential scanning calorimetry; SLS: Static light scattering; Ts-CD: Tosylated-Cycloextrin.

REFERENCES