PREPARATION AND CHARACTERIZATION OF TRIPOLYPHOSPHATE-CHITOSAN NANOGEL AS A DOXORUBICIN CARRIER

Fereshteh Atabi[1], Seyed Latif Mousavi Gargari[2]*, Mehrdad Hashemi[3], Parichehr Yaghmaei[1]
[1] *Department Of Biology, Science and research branch, Islamic Azad University, Tehran, IRAN*
[2] *Department Of Biology, Faculty of Science, Shahed University, Tehran, IRAN*
[3] *Department Of Genetics, Tehran Medical Sciences branch, Islamic Azad University, Tehran, IRAN*
* Corresponding author: Prof. S. L. Mousavi Gargari Tel.: +989123118510, fax: +982151212201,
E-mail address: slmousavi@shahed.ac.ir

ABSTRACT:
Due to their compatibility, degradation behavior, and nontoxic nature on administration, biopolymers are promising materials in drug delivery. Nanostructured drug carriers allow the delivery of not only small-molecule drugs but also of nucleic acids and proteins. Chitosan (CS) a versatile biopolymer of the aminoglucopyran family, the only natural cationic polysaccharide is being extensively explored for various biomedical and pharmaceutical applications. In this study phosphorylated chitosan nanoparticles (PCS) were prepared and loaded with Doxorubicin (DOX) as PCS-DOX for the first time. PCS were prepared by reacting CS with TPP as a crosslinking agent in an emulsion system at tripolyphosphate (TPP): CS ratio of 1:9. The nanogel structure and its derivatives were characterized by Scaning Electron Microscopy (SEM), Fourier-transform infrared (FT-IR), and Dynamic Light Scattering (DLS). Centrifugation and dialysis based loading and releasing capacity of the nanohydrogel was also tested. The average size of PCS particles was 60-160nm. The nanogels have spherical shape, dense surface and homogeneous matrix. The drug loading content increased with DOX concentration and the loading efficiency increased to about 87-97% within 24 h. Approximately 26% of DOX were released from nanoparticles within the first 3 hours. The results obtained in this study suggest that our PCS nanogel with a high drug loading capacity could serve as a proper drug carrier for treatment of cancerous cells.
KEYWORDS: Chitosan, Doxorubicin hydrochloride, Drug Delivery, Loading, Release, TPP.

INTRODUCTION

Nanotechnology with its increasing exploration in different fields of science , making significant advances in biomedical applications such as new drug delivery techniques[1]. Nanotechnology and polymers have captivated a tremendous interest in many areas such as the pharmaceutical industry and therapeutic innovation among others[1]. Typically, a drug-loaded nanocarrier can afford protection against drug degradation or inactivation enroute to the target site[2]. Nanoparticle mediated site specific targeting approaches also offer an opportunity for using some of the most drugs for disease treatment that otherwise could not be administered due to severe drug related toxicity problems[3]. There has been considerable research in the development of biodegradable nanoparticles as effective drug delivery systems[3]. Drug immobilization on a suitable bed is achieved by fixing drugs within supports, offers a proper method for stabilizing, storage and sustained release of drugs. The drug is dissolved, entrapped, adsorbed, attached or encapsulated into the nanoparticle matrix. The nanoparticle matrix can be of biodegradable materials such as polymers or proteins[3]. Depending on the method of preparation, nanoparticles can be obtained with different properties and release characteristics of the encapsulated therapeutic agents[3]. Recently immobilization technics have been significantly improved[4]. Parameters such as nanoparticle size, morphology and chemical makeup can be tailored in relation to the type, developmental stage and location of a given disease[2]. Nanogels are attractive vehicles for drug scavenging in vivo because of their easy injectability, large surface area-to-volume ratios, and low probability of embolic phenomena[5]. While the loading capacity of the current developed nanoscaled drug carriers toward the drugs is still low, therefore, for efficient drug action, improving the loading efficiency is critical in drug carrier research[6]. Chitosan as a nanogel is a natural cationic biopolymer (pKa 6.5-7.0) of glucosamine (GlcN) and N-acetylglucosamine (GlcNAc), obtained by partial deacetylation of acetamide groups of chitin[7,8]. Acetylated and deactivated monomers have been specified to distribute randomly[7,8]. The average molecular weight of the CS is between 10 and 1000 KDa and the degree of deacetylation (DD) is usually between 70 and 95% and are dependent on deacetylation conditions[9]. Chitosan is a natural biodegradable and biocompatible polymer which has been used in controlled drug delivery system[10,11]. CS is generally considered non-toxic, with an oral LD_{50} in mice over 16 g/kg[3]. It is a renewable polysaccharide, easily available in nature with high content of the functional group, low immunogenicity, large surface area for bio-conjugation, low

manufacturing cost and has been used in biomedical areas in the form of sutures, wound healing materials and artificial skin[12,13]. It has been widely studied in the preparation of nanoparticles for drug delivery[7,14-21]. Due to the presence of amino group at the C-2 position of D-glucosamine residues in the CS backbone, the polysaccharide is converted into polycation in acidic media[8,16]. Therefore, micro and nanoparticles can be easily prepared by the electrostatic interactions between the amine groups of CS and a variety of biocompatible polyanionic materials such as citrate, sulfate, tripolyphosphate and *etc.*[16, 17, 20]. These interactions need unique moderate conditions in terms of temperature and pH[19,20]. Chitosan colloids are under extensive investigation mainly for oral, vaginal, and parenteral delivery of many drugs (from low molecular weight compounds to macromolecular drugs), in order to improve the bioavailability of degradable substances such as proteins, or to enhance the uptake of hydrophilic substances across the epithelial layers[13,18]. Prostate cancer is the most common malignancy affecting men worldwide. DOX is currently the most effective drug of choice, therefore in vitro drug loading in and release from the carrier are to be monitored using DOX as a model drug. In the present study, partially phosphorylated CS was used for the preparation of PCS nanogel beads using three polyphosphate (TPP) to improve the controlled release system. Herein, we report a novel noncovalent nanohybrid PCS-DOX and its in vitro loading and release was investigated on the bases of PCS: DOX ratio. We focused on the characterization of the PCS nanogel beads by morphological observation and structural studies by Scanning Electron Microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), and Dynamic Light Scattering (DLS).

MATHERIALS AND METHODS

2.1. Materials

CS and TPP were from Sigma Aldrich (USA). Glacial acetic acid (100%) was from Merck (Germany). DOX was from EBEWE PharmaGes (Austria). All other chemicals used in this research were of analytical grade from Merck (Germany).

2.2. Preparation of TPP-Chitosan (PCS) nanoparticles

CS nanoparticles were prepared following the procedure described by Taranejoo et al.[20] with some modifications. Briefly, 0.1g CS was dissolved in 20 ml of 1% acetic acid with continuous stirring at room temperature (RT) to obtain 5mg/ml homogeneous viscous CS solution. 0.01 g TPP was dissolved in 20 ml deionized water (DW) to obtain 0.5 mg/ml TPP solution and the pH was adjusted to 4.0. TPP solution was added drop wise to 20 ml of CS solution under the sonication power of 70W with continuous stirring for 60 min at RT to obtain a TPP: CS ratio of 1:9. The mixture was stirred continuously for another 2 hours and was then labeled as S1 and stored at 4 ^{o}C. 10 ml of S1 was centrifuged at 4000 ×g for 15 min at 18 ^{o}C. The supernatant was discarded and the precipitated nanoparticles were washed twice with DW. The pellet was suspended in DW and sonicated to obtain 0.5% PCS. The PCS labeled as S2, was then stored at 4 ^{o}C. One ml aliquots of the mixture were lyophilized with a freeze dryer, labeled as S3 and stored at RT. One ml of DW was added to the lyophilized nanogel and sonicated at 70W until completely dissolved.

NANOGEL CHARACTERIZATION

3.1. Particle size measurement

The particle size of the nanogels was determined by Scanning Electron Microscopy (SEM) and related standard deviation were calculated for all the three samples. The microparticles were sputter-coated with a thin layer of gold and scanned by KYKY-EM3200 SEM at 25 KV accelerating voltages. The mean particle size and particle size distributions of the nanogels were also determined by Zeta Plus Dynamic Light Scattering (DLS) Zeta Sizer Nano-ZS-90 (Malvern Instruments). Particle size was measured as mean of more than 30 replicates and Poly dispersity Index (PdI) were calculated. Prior to mean particle size measurement, samples were dissolved in DW, 0.5wt. % related to the dry mass of nanogel in the mixture and were sonicated in a water bath to disperse the nanoparticles. The electrophoretic mobility of the PCS nanogels was also measured using the Zeta Plus instrument operating in the mode of Phase Analysis Light Scattering (PALS).

3.2. FT-IR test

CS, PCS, DOX and PCS-DOX were characterized by Fourier-transform infrared spectroscopy (FT-IR). Spectral analyses were recorded by a spectrophotometer of Thermo Nicolet Nexus 870 FT-IR ESP (USA). About 100 mg from each dry sample of CS, PCS, DOX and DOX-PCS were ground thoroughly with KBr and tablets were prepared under a pressure of 600 kg/cm^2 using a hydraulic press. At least three tablets were prepared for each sample. Tablets were used for FT-IR analysis.

3.3. Centrifugation-based Drug loading and releasing

Centrifugation-based drug loading and release was determined as described by Taranejoo et al.[20] 5 mg freeze-dried PCS nanoparticles were mixed with 1 ml from two separate concentrations (2 and 5 mg/ml) of DOX in phosphate-buffered saline (PBS), pH 7.4. The reaction mixtures were sonicated and incubated for 24 h/4 ^{o}C under

shaking condition. The samples were then centrifuged at 13000×g for 20 min. The supernatants were removed and the pellets were dissolved in 1.5ml PBS by sonication and incubated for 72h /37 °C under shaking condition. At various time intervals the mixtures were centrifuged and 0.7ml of each supernatant was removed and analyzed by High-performance Liquid Chromatography (HPLC) for the presence of DOX and replaced with the same quantity of PBS. The pellets were dissolved by sonication and incubated at 37 °C.

3.4. Dialysis-based drug loading and release

Dialysis-based drug loading and release was also determined using the method of Wang et al.[23]. To determine the influence of PCS concentration on DOX loading and release, 0.5, 0.8 and 1.0 mg of PCS nanogel were added to 0.5ml at DOX concentration of 2mg/ml. The solutions were mixed thoroughly for 24h/4 °C under shaking condition. In order to remove the free DOX, solutions were dialyzed using 3kDa MWCO dialysis bags against 30 ml DW for 3 hours at 37°C. The DOX loaded PCS was then dialyzed against 30 ml of phosphate-buffered saline, pH 7.4 at 37 °C. At various time intervals 2 ml of phosphate-buffered saline from each dialysis buffer was taken and analyzed by HPLC for the presence of DOX. The buffer taken for analysis was replaced with an equal volume of fresh buffer.

3.5. Determination of loading capacity and release rate of DOX

The amounts of the loaded and released DOX were determined using reverse-phase High-performance Liquid Chromatography (HPLC) according to the method given by EBEWE PharmaGes Company. The concentration of DOX was calculated using a standard curve of DOX. 50, 75, 100, 125 and 150mg/L concentrations of DOX were injected to a reversed phase column (C18, 5µm, 250mm × 4.6mm) and eluted with sodium lauryl sulfate-Phosphoric acid: acetonitrile of 50:50 ratio with a flow rate of 1 ml/min. The OD of DOX was read at 254 nm. The sample solutions were detected with a run time of 7 min for assay.

The loading efficiency (LE) and loading capacity (LC) of the drug were calculated according to the following equations:

LE = (Total amount of DOX − Free DOX) /Total amount of DOX

LC = (Total amount of DOX − Free DOX) /Amount of PCS

RESULTS AND DISCUSSION

4.1. CS nanogels and particle size

CS is generally considered to be a non-toxic[3,24], biodegradable, biocompatible with an oral LD_{50} of over 16 g/kg in mice[3]. One of the most convenient and effective approaches to modify the properties of CS hydrogels is introduction of crosslinking in CS. The reactive sites for crosslinking reactions have been provided by the hydroxyl and amino groups on glucosamine units in CS. The TPP treatment of CS nanoparticles can improve their stability and applicability in controlled drug delivery. Following treatment with TPP, the positive charge of chitosan is converted to a negative one, favoring its strong electrostatic interaction with mucus or a negatively charged mucosal surface, making it a good drug-delivery system for the selective delivery. The micro and nanoparticle size can be controlled by changing the CS: TPP ratio, pH and their molar mass[21, 25, 26]. In the present study, a novel method for nanogel synthesis was used to produce nanoparticles based on CS and TPP at different ratios. Table 1 and Figure 1. show the results of the particle size of PCS nanogels characterized by SEM. The particles were of average diameter of about 65, 116 and 104 nm for S1, S2 and S3 respectively. The Zeta–potential of the nanoparticles determined for three samples in DW/25 °C are given in Table 1 and Figure. 2(a-d). The average size of the particles obtained by Zeta potential were 69, 152 and 128nm for S1, S2 and S3 respectively. The size distribution for the three prepared PCS nanogels were found to be 40–150 nm (Figure 2a). The polydispersity obtained for S1, S2 and S3 were 0.184, 0.406 and 0.312, respectively (Table 1). The SEM images also confirmed the same size range of about 60–160 nm for the CS nanogels (Figure 1a-c) having a spherical morphology with dense surface and a homogeneous matrix. Using the lower ratio of TPP leaves most of the positively charged amino groups of CS, which can then be interacted with other reactive groups such as glutaraldehyde, carboxyl-Poly Ethylene Glycol, and *etc*., for drug delivery. The best TPP: CS ratio was found to be 1: 9 in our work. Being a cross linker, TPP concentration also plays a crucial role in the particle size and particle aggregation[20, 25]. In addition the 1:9 ratio of TPP: CS makes the polymer soluble at physiological pH, whereas the higher TPP concentrations make the polymer insoluble. The CS concentration used in our research was 5 mg/ml and the small particle size can be attributed to the low concentration of CS. Tsai et al.[21] also demonstrated that increasing CS concentration could result in increasing the mean diameter of nanoparticles.

Table 1: SEM and DLS of PCS nanogels. S1: mixture of TPP: CS at a ratio of 1:9, stirred 2 hours, S2: pellet obtained from centrifugation of S1 and then dissolved in DW to obtain 0.5% PCS, S3: lyophilized form of S2.

PCS	S1	S2	S3
Mean Particle size	65±20.38	116±32.65	104±39.11
DLS Record Number	43	36	46
Zeta potential (mV)	28.7±7.02	28.7±7.02	25.6±7.10
Z-Average size (d. nm)	69.46	152.2	128.35
PdI	0.184	0.406	0.312

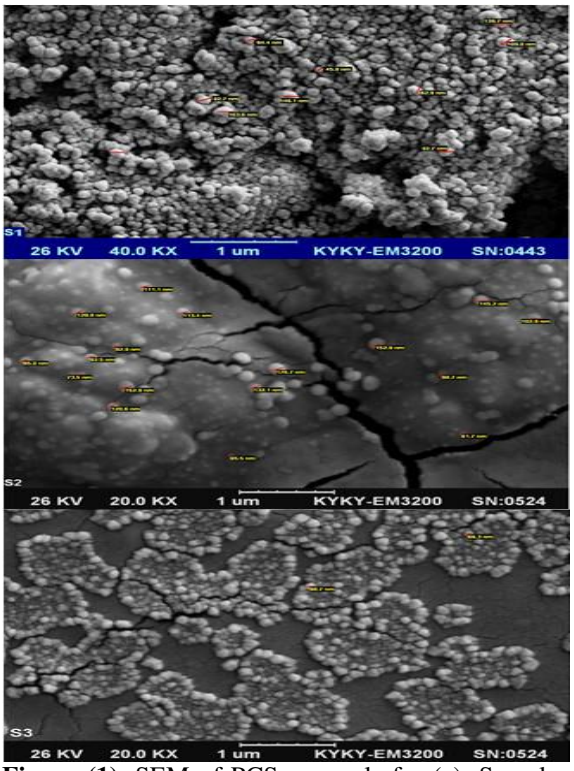

Figure (1). SEM of PCS nanogels for (a). Sample S1, (b). Sample S2, and (c). Sample S3

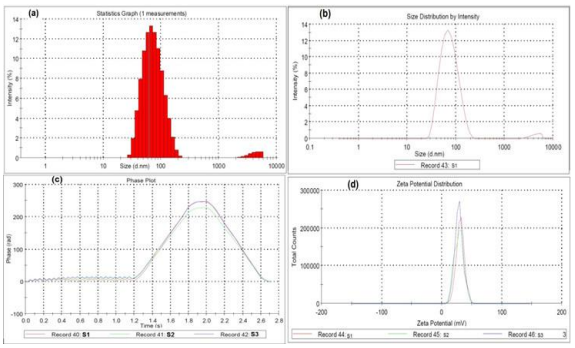

Figure (2): DLS Results for PCS nanogels (a, b, c, d). (a). Statistics Graph of S1. (b). Size Distribution Report by Intensity. (c). Comparison of phase plots of the S, S2 and S3. (d). Comparison of Zeta Potential Distribution of S1,S2 and S3 .

4.2. In vitro Drug loading and release

In order to study the effect of DOX concentration on loading, different amounts of DOX were incubated with the PCS nanogels. The drug loading capacity was increased with increasing DOX concentration, whereas the loading efficiency didn't increase beyond 1:1 ratio of PCS: DOX. The 24 h incubation time enhanced the loading amount of DOX to about 97% (Table 2 and Figure 3a). At the constant amounts of DOX, the drug loading partially increased with increasing PCS concentration (Table 3). Jayakumar et al.[24], obtained the loading efficiency of about 26% in Chitin-DOX within 5 h. They reported that the only factor affecting the loading efficiency is increasing the incubation time. But the size of the Chitin loaded DOX nanogels remained constant because of the surface adsorption of the drug[24]. Taranejoo et al.[20] demonstrated that other factors such as the concentration of CS and the amount of TPP, drug polymer ratio and stirring speed may affect the loading efficiency of drug or biological agents in PCS. Shahsavari et al.[27], also reported that, decreases in the concentration ratio of chitosan/TPP consequently cause an increase in entrapment efficiency. Data from in vitro centrifugation-based drug release studies indicated approximately 26% of DOX release from nanoparticles within the first 3 hours, followed by slow release to reach approximately to 35% after 120h. The results of in vitro dialysis-based drug release with different PCS concentrations is shown in Figure 3(b). Data obtained from in vitro dialysis-based drug release showed approximately16. 5% DOX release within the first 3 hours and reached to 28% after 120 h. The release profile of DOX / 37 °C was time dependent and the pattern of release was similar to that of centrifugation-based release. Similar results are reported by Yang et al.[6]. They showed 11% of DOX release from graphene oxide after 5 h under neutral conditions (pH 7). But the release percentages of DOX reported by Xu et al.[26] were relatively fast (73.1%, 60.5%, 47.7% and 36.8%) after 10 h for the four samples. They also showed that the DOX release behaviors are closely related to the distribution status of the DOX content and its concentration during the encapsulation. Slow release of DOX molecules out in an aqueous phase from polymeric conjuncts, could provide continuous release of DOX out in the tumor tissue. This sustained release of DOX at the target site would take an additional advantage in decreasing the tumor volume[28].

Table 2: Centrifugation-based drug Loading of PCS-DOX

DOX (mg/ml)	PCS:DOX	Loading Capacity (DOX/mg PCS)	Drug Loaded (mg/L)	Loading Efficiency (%)
2	1 : 0.4	346	1730	85.5
5	1 : 1	883	4413	88.3

Table 3: Dialysis-based drug Loading of PCS-DOX

PCS mg	PCS:DOX	Loading Capacity (DOX/mg PCS)	Drug Loaded mg/L	Loading Efficiency (%)
0.5	1 : 2	1930	965	96.5
0.8	1 : 1.25	973	973	97.3
1.0	1 : 1	650	975	97.5

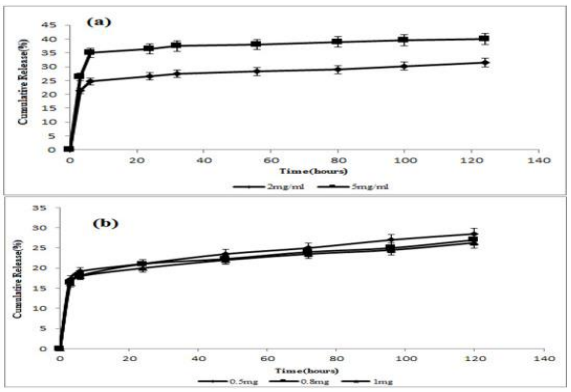

Figure (3): Sample release during 80h. (a). Release of samples with 2 and 5 mg/ml loaded DOX. (b). Release of samples with different amounts of PCS.

4.3. FTIR Analysis

The structural characterizations of non-modified CS, DOX, PCS, and DOX-PCS evaluated with FT-IR spectrometry are shown in Figure 4(a-d). The peak at 1601 cm^{-1} could be attributed to N-H bending, at 1158 cm^{-1} belongs to C–O–C in glycosydic linkage and 1661 cm^{-1} is due to C=O carbonyl stretching vibrations of the esteemed group in CS (Figure 4a). The non-sharp characteristic peak at 2867 cm^{-1} is due to the C–H stretching vibrations of methyl or methylene group. The stretching vibration of the O-H group is overlapped to the N-H stretching band at 3367 cm^{-1} to give a very strong and broad peak in non-modified CS. The stretching vibration of the O-H group is overlapped to the N-H stretching band at 3367 cm^{-1} to give a very strong and broad peak in non-modified CS. Similar results were also reported by Mitra et al.[29]. The peaks of at 2897 cm^{-1} (C–H stretching vibrations), 779 and 701 cm−1 (N–H wagging), 3382 cm^{-1} (stretching of alcoholic O–H groups), 1416 cm^{-1} (C–C stretching), 779 cm^{-1} (NH2 and N–H wagging) are characteristics of DOX (Figure 4b). The peaks at 1144 cm^{-1} and 1089 cm^{-1} in the spectrum of the PCS-DOX are referring to the P=O and the P–O–R groups, respectively (Figure 4d). The peaks at 592 cm^{-1}, and 658 cm^{-1} are because of O–C–O scissors present in CS structure and is moved to 893 cm^{-1} in PCS formed by the phosphorification reaction to form P–O–R esters. The DOX peak at 1220 cm^{-1} was seen in DOX loaded PCS nanogels confirming the presence of DOX in the PCS nanogels (Figure 4b and d). The results reported by R. Jayakumar et al.[24] *S.* Kayal et al.[30] are in support of our findings. A shift from 779 to 887 cm^{-1} related to N–H wagging, is due to the presence of intermolecular hydrogen bonding between the DOX and PCS loaded with DOX. Following the chemical modification of CS, the absorption band at 1601 cm^{-1} is moved to a lower position at 1550 cm^{-1} after PCS formation, and 1661 cm^{-1} is shifted to 1647 cm^{-1} in the PCS (Figure 4c) and to a higher position at 1674 cm^{-1} in PCS-DOX nanohybrid (Figure 4d). This could be attributed to the DOX loading in PCS and the shift of characteristic peaks may be due to the hydrogen bonding between these two components. A similar result has been observed for the graphene oxide- DOX nanohybrid by Yang et al.[6]. The absorption band at 2133 cm^{-1} in CS is shifted to 2158 cm^{-1} in PCS-DOX and a new band at 2330 cm^{-1} has also been created in PCS-DOX (Figure 4d). The C–O stretching absorption peak of the secondary hydroxyl group at 1098 cm−1 in CS became stronger and was shifted to 1075 cm^{-1} in the PCS (Figure 4c). The characteristic peaks of the PCS-DOX conjugates from the N–H stretching band at 3389 cm-1 and O–H stretching band at 3632 cm^{-1} are overlapping at 3390 cm^{-1} in the PCS and at 3367 cm^{-1} in CS were also confirmed by FT-IR spectroscopy (Figure 4a, c and d). FT-IR spectrum of the PCS-DOX nanohydrogel shows several absorption bands at 511-887 cm^{-1} which have been shifted to 586-779 cm^{-1} from DOX, also confirming the presence of DOX in the PCS nanogels (Figure 4b and d).

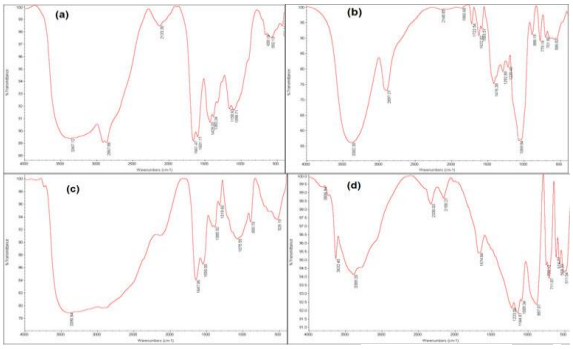

Figure (4): FTIR spectra of (a). Non-modified CS, (b). DOX, (c). PCS, and (d). DOX loaded PCS.

CONCLUSION

Our simple PCS-DOX formulation can be replaced with complicated procedures used for the preparation of the same kind. Availability, non-toxicity, biodegradablity, biocompatibility and low

immunogenicity of the renewable polysaccharide i.e. CS are attractive features of this nanoparticle for drug delivery. High loading capacity with a low releasing slope of DOX from PCS suggest that PCS nanogels could be an alternative carrier as a cancer therapeutic agent.

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