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Preparation of low toxic fluorescent chitosan-*graft*-polyethyleneimine copolymer for gene carrier

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ABSTRACT

Fluorescent chitosan-*graft*-polyethyleneimine (PEI) copolymer was prepared by incorporating PEI molecule onto chitosan backbone through naphthalimide moiety by simple substitution reaction. 4-Bromo-1,8-naphthalic anhydride was used as fluorescent probe due to its strong fluorescence and good photo-stability property and the presence of a fine tunable bromide functional group in the naphthalimide ring, in this work. The copolymer was characterized by FTIR, elemental analysis and XRD. The fluorescence property of the copolymer was determined by UV–vis spectrometer and spectrofluorometer. The effects of pH and temperature on fluorescence property of the copolymer were also studied. The graft copolymer with degree of substitution 37.6 of PEI onto chitosan showed better complexation ability with DNA at comparatively low N/P (nitrogen to phosphate ratio) ratio 1.0 compared to that of chitosan (N/P ratio 2.0). The cytotoxicity of PEI largely decreased after grafting with chitosan and all the copolymers showed above 50% cell viability even at high polymer concentration (300 μg/mL). Therefore, the prepared fluorescent chitosan-*graft*-PEI copolymer may be used as a biological marker as well as drug or gene carrier with low toxicity.

1. Introduction

Recently, fluorescent polymers have attracted much attention due to their captivating structure as well as potential applications as functional materials in many areas including pH sensing, chemosensors, electroluminescent devices, nuclear detection, biological markers in biomedical field and so on. Fluorescently labeled polymers provide information about locations and dynamics that can be impossible or very difficult to obtain using unlabeled materials. In the last decade, various fluorescent polymers such as poly (vinyl carbazol) (Zhang et al., 2008), rhodamine 6G-tagged poly (vinyl alcohol) (Zondervan, Kulzer, Orlinskii, & Orrit, 2003) poly (α-vinyl-ω-alkyloligothiophene) (Melucci et al., 2004) and fluorescent chitosan-gelatin bioconjugate (Mi, 2005) have been prepared. Fluorescent polymers from natural materials for biomedical application are becoming favorable candidates due to their biodegradability and biocompatibility.

Among the different fluorescent dyes, naphthalimide fluorescent dyes have strong fluorescence and good photo-stability property and they have found application such as biology markers (Fei & Gu, 2009), anticancer agents (Ott et al., 2008), analgesics in medicine (de Souza, Correa, Filho, Grabchev, & Bojinov, 2002), fluorescent sensors (Parkesh, Lee, & Gunnlaugsson, 2007), light emitting diodes (Gan, Song, Hou, Chen, & Tian, 2004), electroluminescent materials (Ding et al., 2008) and so on. Among the naphthalimide derivatives, 4-Bromo-1,8-naphthalic anhydride dye is one of the most important derivatives because of its most important yellow components for day light fluorescent pigments.

Chitosan (CTS), isolated from chitin by alkaline hydrolysis, is the linear and partly acetylated (1→4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977; Muzzarelli et al., 2012). Chitosan has gained considerable attention as a gene delivery vector (Kundu & Sarkar, 2011; Lee, 2007; Mao, Sun, & Kissel, 2010; Muzzarelli, 2010a; Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011) due to its favorable biological properties such as low toxicity, good biocompatibility and biodegradability (Kean & Thanou, 2010; Muzzarelli, 2010b). Chemical modifications introduce hydrophilic groups into chitosan, the most common ones being quaternization (de Britto, Goy, Filho, & Assis, 2011; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009), carboxymethylation (Muzzarelli, 1988) and grafting of poly(ethylene glycol) (Deng, Qi, Yao, Feng, & Dong, 2007).

On the other hand, polyethyleneimine is a highly cationic and water soluble polymer and is most widely used non viral vector in gene therapy due to its high transfection efficiency (Sonawane, Szoka, & Verkman, 2003; Thomas & Klibanov, 2002). But, its severe toxicity limits their clinical application. It is well known that chitosan is a nontoxic biopolymer and it has unique glucosamine

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repeating units in its polysaccharide backbone. Due to the presence of primary amine group in its backbone, chitosan in acidic solution is cationic, which helps it to encapsulate and deliver the genetic materials due to electrostatic interaction. Therefore, the chemical combination of chitosan with PEI may provide novel biomaterial with improved water solubility, higher charge density and lower toxicity. Previously, polyethyleneimine has been modified with various materials such as, serum albumin (Rhaese, Briesen, Rübsamen-Waigmann, Kreuter, & Langer, 2003), dextran (Tseng & Jong, 2003), hydrophilic polyethylene glycol (PEG) (Chen et al., 2011) to improve biocompatibility and reduce cytotoxicity. One alternative way to reduce the toxicity of PEI is to use lower molecular weight PEIs. However, low molecular weight PEIs are effective in binding pDNA but their gene transfection efficiency is limited both in vitro and in vivo (Godbey, Wu, & Mikos, 1999; Gosselin, Guo, & Lee, 2001; Kunath et al., 2003). Therefore, our aim of this work to prepare chitosan-*graft*-PEI copolymer with reduced toxicity and the obtained compound can be used as biological marker. Here, we first prepared N-(4-bromonaphthalimide)-chitosan and then, coupled the PEI molecule with chitosan through naphthalimide moiety by simple substitution reaction. The fluorescence property of the copolymer was evaluated by UV–vis spectrophotometer and spectrofluorometer. Enhanced DNA complexation property of the copolymer due to increased cationic property of chitosan by the incorporation of PEI molecule onto chitosan backbone was confirmed by agarose gel electrophoresis and ethidium bromide assay. In vitro toxicity of the copolymer was carried out by MTT assay on HeLa cell line. In vitro DNA release from self-assembled copolymer/DNA complex was also studied.

2. Experimental

2.1. Materials

Chitosan (MW-75 kDa and DDA 69%) and 4-bromo-1,8-naphthalic anhydride were purchased from Sigma–Aldrich. Specially dried DMSO was obtained from Merck, India. pGL3 control vector (5.25 kb) containing SV-40 promoter, resulting in strong expression of luc$^+$, was purchased from Promega (Madison, WI, USA). The plasmids were propagated in *Escherichia coli* (*E. coli*) and the plasmid DNA (pDNA) was isolated with QIAGEN Midiprep pDNA isolation Kit (USA) according to the manufacturer's instructions. Its purity was confirmed by spectrophotometry (A_{260}/A_{280}) and its concentration was determined from its absorbance at 260 nm. All other reagents were analytical grade and were used directly without further modification.

2.2. Deacetylation of chitosan

To increase the amine group content of chitosan, it was further deacetylated according to previous method (Yuan, Chesnutt, Haggard, & Bumgardner, 2011). Briefly, 10 g chitosan was treated in 50% NaOH solution (by weight) for 1 h in a round bottom flask at 110 °C under nitrogen atmosphere. The reaction mixture was subsequently neutralized by hydrochloric acid. Then, the product was filtered and washed with distilled water for few times and finally dried under vacuum at 50 °C for 24 h.

The degree of deacetylation (DDA) of purchased chitosan and deacetylated chitosan was determined by potentiometric titration according to our previous method (Sarkar, Srivastava, Chatterjee, & Kundu, 2011). The DDA (%) of CTS was calculated by using the following equation:

$$\mathrm{DDA}(\%) = \frac{203.2}{42.0 + \frac{1000m}{C_{\mathrm{NaOH}}(V_2 - V_1)}} \times 100$$

where m is the amount of chitosan (g) in the solution, C_{NaoH} is the concentration of NaOH solution (M), $(V_2 - V_1)$ is the volume of NaOH solution consumed in titration of amino groups of chitosan, 203.2 is the molecular weight of the acetylated monomeric unit of the polysaccharide, 42.0 is the difference between the molecular weights of the deacetylated monomeric unit and acetylated monomeric unit, 1000 is the conversion factor of milliliters to liters and 100 is the conversion factor of DDA to percents.

2.3. Preparation of N-(4-bromonaphthalimide)-chitosan **3**

N-(4-bromonaphthalimide)-chitosan was prepared according to the previous method (Munro, Hanton, Robinson, & Simpson, 2008) with slight modification. Briefly, 1 g chitosan (MW-70 kDa and DDA 78%) was suspended into 50 mL DMSO containing different amounts of 4-bromo-1,8-naphthalic anhydride **2** (chitosan:4-bromonaphthalic anhydride mol ratio-1:0.5, 1:0.25 and 1:0.125) and the reaction mixture was refluxed at 80 °C under nitrogen atmosphere for 3 h. Then, the reaction mixture was filtered in hot condition and washed with hot DMSO to remove unreacted 4-bromonaphthalic anhydride. Finally, the product was washed successively with water, methanol and acetone and then vacuum dried at 50 °C for 24 h to get brown colored compound **3**.

2.4. Preparation of N-naphthalimide chitosan-graft-PEI **4**

N-naphthalimide chitosan-graft-PEI copolymer was prepared by heterogeneous method by the following procedure. 100 mg of compound **3** was dispersed in 50 mL aqueous solution of PEI with different concentrations (5%, 15% and 30%) to prepare different substituted N-naphthalimide chitosan-graft-PEI (NCTS-graft-PEI) copolymer. The reaction mixture was then refluxed at 80 °C under nitrogen atmosphere for 3 h. The product was filtered and washed with water, methanol and acetone. Finally, the product was dried under vacuum at 50 °C for 24 h to get yellow colored compound **4**.

2.5. Characterization of the polymer

Fourier transform infrared (FTIR) analysis was carried out with ATR FTIR spectroscopy (model-Alpha, Bruker, Germany). The sample was uniformly mixed with potassium bromide at 1:10 weight ratio and KBr pallets were prepared using 10 ton hydraulic pressure for 10 min at room temperature. Then, FTIR spectra of the pallets were carried out within the frequency range of 4000–500 cm^{-1} for 42 consecutive scans.

The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were determined on Bruker AV 3000 Supercon NMR system (Germany) at 300 MHz using D_2O/DCl and D_2O as solvent. Chemical shifts (δ) were reported in ppm using tetramethylsilane (TMS) as an internal reference.

The elemental analysis was performed using 2400 series II CHN Elemental Analyzer (Perkin Elmer). The percentage of carbon, nitrogen and hydrogen was estimated.

X-ray diffraction spectrometry of the polymers in the powder form were performed by a wide angle X-ray scattering diffractometer (Panalytical X-Ray Diffractometer, model- X'pert Powder) with Cu K_α radiation ($\lambda = 1.5444$) in the range 5–35° (2θ) at 40 kV and 30 mA.

UV absorbance spectra of chitosan and compound **4c** in acetic acid/sodium acetate buffer were measured by UV–vis spectrophotometer (OPTIZEN POP BIO, Mecasys) using nano liter cell. The effect of pH on UV absorbance was performed with compound **4c**.

Fluorescent spectra were measured with JASCO FP-8000 Spectrofluorometer in acetic acid/sodium acetate buffer. The effects of

pH and temperature on fluorescence intensity were also performed with compound **4c**.

2.6. Preparation of N-naphthalimide chitosan-graft-PEI/pDNA complex

Polymer/DNA complexes were prepared with by a complex coacervation method. Both chitosan (CTS) and compound **4c** were dissolved in acetic acid/sodium acetate buffer at pH = 5.5 with a concentration of 2 mg/mL and the solutions were filtered by Millipore 0.45 μm filter paper. pDNA was dissolved (100 μg/mL) in 25 mM of sodium sulfate solution. The polymer solutions were then diluted with buffer to get the desired concentration for polyplex formation. Both the polymer and DNA solutions were preheated separately at 50–55 °C for 10 min. Then, polyplexes at various charge ratios (amino group to phosphate group ratio, N/P ratio) were prepared by immediately mixing of equal volume of polymer and pDNA solution and subsequently vortexing for 15–30 s with cyclomixer (REMI, India). After that, the resulting mixtures were incubated at room temperature for 30 min for complete formation of polyplexes.

2.7. Agarose gel electrophoresis assay

The binding ability of polymer with pDNA was determined by agarose gel electrophoresis. Agarose gel (0.7%, w/v) was prepared in TAE buffer (40 mmol/l Trisacetate, 1 mmol/l EDTA) and ethidium bromide (10 μg/ml) was added to the gel as a DNA visualizer. Then, the samples with different N/P ratios were loaded in the gel for electrophoresis. The gel electrophoresis was carried out at 100 V for 45 min. The picture of the gel was subsequently captured by BIOTOP gel doc system (Shanghai, China).

2.8. Ethidium bromide displacement assay

Ethidium bromide displacement assay was carried out according to our previous report (Sarkar et al., 2011). Briefly, a solution of pDNA (10 μg/mL) in HEPES buffer was mixed with ethidium bromide (EtBr) in molar ratio EtBr/DNA phosphate of 1:10. DNA-chitosan/chitosan derivative complexes were formed with different N/P ratios directly in the 3 mL cuvette by stepwise addition of polycation to the DNA solution. The fluorescence of samples was measured 5 min later after addition of polycation at excitation and emission wavelengths of 493 and 523 nm, respectively (JASCO FP-8000 Spectrofluorometer). The recorded fluorescent intensities were expressed relative to the fluorescence intensity of the DNA-EtBr solution in the absence of polycation, after subtracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions.

2.9. Determination of DNA loading efficiency

Equal volume of polymer solution in 5 mM sodium acetate buffer at pH 5.5 and pDNA solution in 25 mM sodium sulphate solution (100 μg/mL) were separately heated at 55 °C for 10 min and immediately mixed at different N/P ratios (0.5, 1.0, 1.5, 2.0, 3.0 and 5.0) and subsequently vortexed for 30 s and incubated at room temperature for 30 min to form polymer/DNA complexes. After complex formation, the solution mixture was then centrifuged and the supernatant was collected to determine the amount of free DNA by UV–vis spectroscopy (OPTIGEN POP BIO, Mecasys Co. Ltd., Korea) at 260 nm. The DNA loading efficiency in percent was calculated by the following equation:

$$\%\text{DNA loading efficiency} = \frac{A - B}{A} \times 100$$

where A = total concentration of DNA (mg/mL) and B = concentration of unloaded DNA (mg/mL).

2.10. In vitro cytotoxicity assay of the polymer

The cytotoxicity of chitosan and graft copolymers was determined by MTT assay. HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 180 μL of growth medium and incubated for 24 h prior to the addition of polymers. Then, 20 μL filtered chitosan, graft copolymers and polyethyleneimine (PEI) as control polymer with final concentrations of 5, 10, 25, 50, 75, 100, 200 and 300 μg/mL, respectively were added to each well. Untreated cells in growth media were used as the blank control. Cells were incubated for 48 h, followed by the addition of 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) in PBS buffer. After further incubation for 4 h, the media was removed and replaced with 150 μL DMSO to dissolve the MTT formazan crystals. The absorbance was recorded at 570 nm by an ELISA microplate reader (Bio-Rad). The cell viability (%) was calculated according to the following equation:

$$\text{cell viability}(\%) = \frac{\text{OD}_{570(\text{sample})}}{\text{OD}_{570(\text{control})}} \times 100$$

where $\text{OD}_{570(\text{sample})}$ represents measurement from the wells treated with polymer and $\text{OD}_{570(\text{control})}$ from the wells treated with DMEM only. All data are presented as the mean of six measurements (±SD).

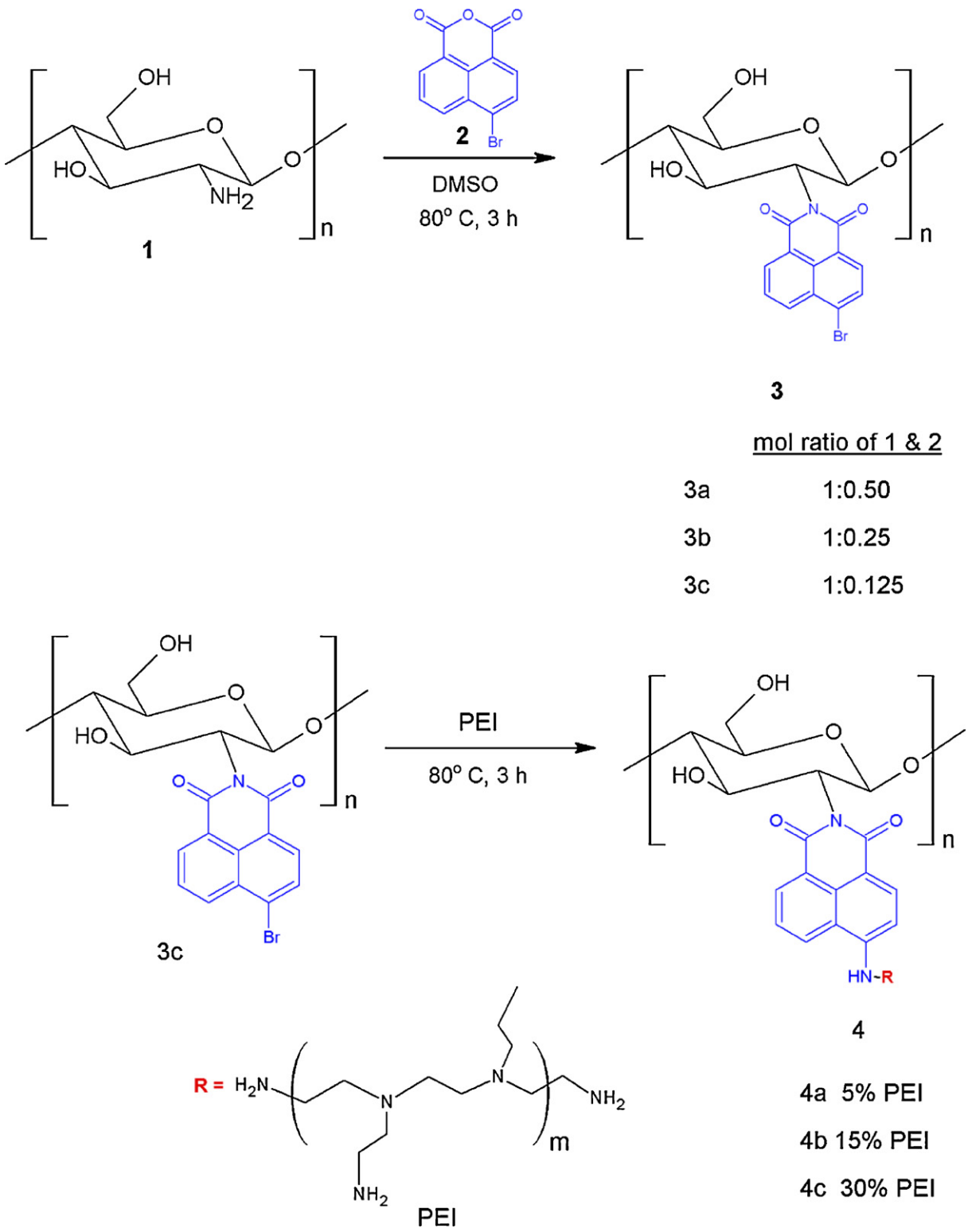
2.11. In vitro DNA release study

In vitro DNA release study was carried out according to our previous report (Sarkar & Kundu, 2012). Phosphate buffered saline buffer (pH 7.4) was used as media for DNA release study. For this study, N-naphthalimide chitosan-*graft*-PEI/DNA complexes at N/P ratio 5.0 were prepared prior to the release study and 300 μL buffer solutions were added to the complexes and incubated in an incubator (Orbit, India) at 37 ± 0.5 °C. At the sampling time, the incubated dispersing solution was centrifuged at 13,000 rpm for 10 min and 250 μL of supernatant was collected for the evaluation of the amount of released DNA using a spectrophotometer at 260 nm. An equal volume of fresh buffer was then replaced the solution and the same procedure was repeated for the next sampling time.

3. Results and discussion

3.1. Preparation and characterization of graft copolymers

Fluorescent chitosan-*graft*-PEI copolymer was prepared by simple substitution reaction through naphthalimide moiety. The synthetic process involved of two steps; preparation of N-(4-bromonaphthalimide)-chitosan (compound **3**) followed by the addition of polyethyleneimine (PEI) to prepare fluorescent chitosan-*graft*-PEI (compound **4**) through substitution of bromine atom of bromonaphthalimide by the primary amine group of PEI. The advantage of the preparation of this copolymer lies in its two stage process where fluorescence functionality can be introduced at the first stage (attachment of naphthalimide moiety to the chitosan) and the attachment of cationic functionality at the second stage (attachment of PEI with chitosan through naphthalimide moiety). The synthetic route of the preparation of fluorescent chitosan-*graft*-PEI copolymer preparation is shown in Scheme 1. FTIR spectra of CTS, naphthalic anhydride, fluorescent chitosan and fluorescent CTS-graft-PEI are shown in Fig. 1. As shown in Fig. 1a, the characteristic peaks of CTS are: 3422.55 cm^{-1} (wide peak of



Scheme 1. Synthetic route of fluorescent chitosan-*graft*-PEI copolymer.

O—H stretching overlapped with N—H stretching), 2926.44 and 2878.68 cm^{-1} (C—H stretching), 1652.98 cm^{-1} (NH—CO (I) stretch), 1597.16 cm^{-1} (N—H bend), 1155.46 cm^{-1} (bridge —O— stretch) and 1095.16 cm^{-1} (bridge C—O—C stretching and C—O stretching). Fig. 1b shows the symmetric and antisymmetric C=O stretches of compound **2** at 1777.16 and 1731.86 cm^{-1}, respectively. After reaction of CTS and compound **2**, the peaks for C=O stretch and N—H bend are changed to 1702.00 cm^{-1} and 1662.67 cm^{-1}, respectively in compound **3c** (Fig. 1c). This fact may be explained by the fact that the environment of some primary amine groups of chitosan has been changed to imide groups. Fig. 1d shows the characteristic peaks obtained for compound **4c** and the peaks are: 3511.35 cm^{-1} (wide peak of O—H stretching overlapped with N—H stretching), 2881.16 cm^{-1} (C—H stretching), 1664.38 cm^{-1} (amide II band, N—H

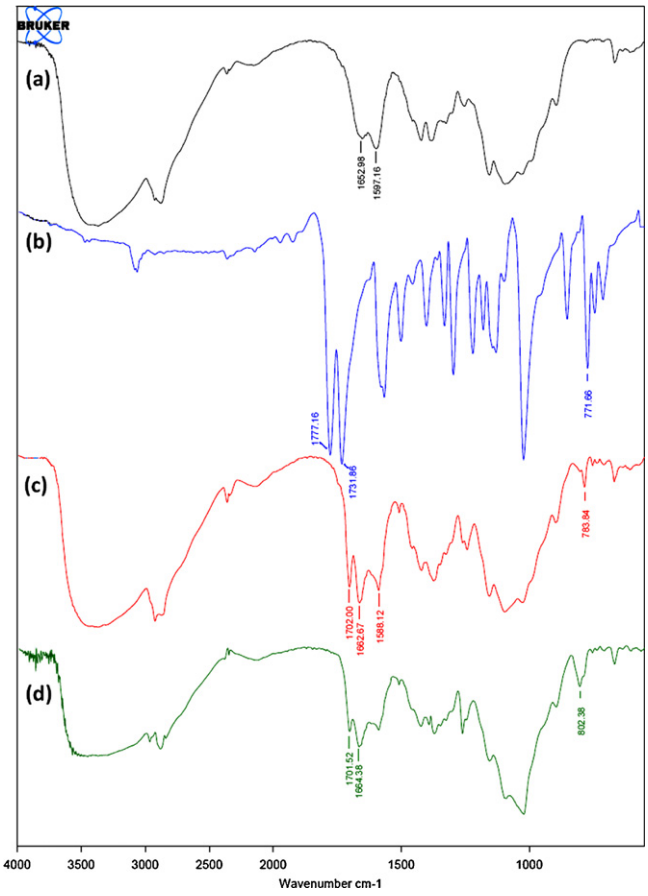


Fig. 1. FTIR spectra of chitosan (a), compound **2** (b), compound **3c** (c) and compound **4c** (d).

bending and C—O stretching of acetyl groups), 1422.57 cm^{-1} (O—H bending, C—N stretching, asymmetric C—H bending of CH_2 group), and 1089.55 cm^{-1} (bridge C—O—C stretching and C—O stretching). The peak at 3422.55 cm^{-1} for CTS has shifted to higher wave number (3511.35 cm^{-1}) and becomes more wider and intense after PEI grafting, indicating that the density of N—H groups in compound **4c** are enhanced after PEI grafting. The intensity of spectral peak assigned for C—H stretching is decreased after PEI grafting and two spectral peaks at 2926.44 and 2878.68 cm^{-1} in CTS reduces to one spectral peak at 2881.16 cm^{-1} in compound **4c**. The other change in spectral peak is noted at 1095.16–1031.20 cm^{-1} after PEI grafting in CTS through naphthalimide moiety. Thereby, FTIR results clearly suggest that PEI is successfully grafted into CTS through naphthalimide moiety in this study.

The ^{1}H NMR spectra of chitosan and compound **4c** are shown in Fig. 2a and b. From Fig. 2a, typical peaks at 3.4–4.0 ppm are assigned to glucosamine unit (H3, H4, H5, H6) of chitosan, the peak at 3.1 ppm is responsible for H2 and the peak at 2.1 ppm is assigned to the methyl protons of N-acetyl group. After the reaction between compound **3c** and PEI (30%), new peaks appear at 7.6–9.0 ppm and 2.7–3.5 ppm in the NMR spectrum of compound **4c** (Fig. 2b), attributed to aromatic protons of naphthalimide moiety and the methylene protons of PEI, respectively. This result indicates that PEI grafted successfully on the chitosan backbone through naphthalimide moiety.

The preparation of fluorescent chitosan-*graft*-PEI copolymer through naphthailmide moiety was further confirmed by elemental analysis. The C, H and N contents of chitosan, compound **3** and compound **4**, determined by elemental analysis, are described in Table 1. As shown in Table 1, the C, H and N contents of chitosan are

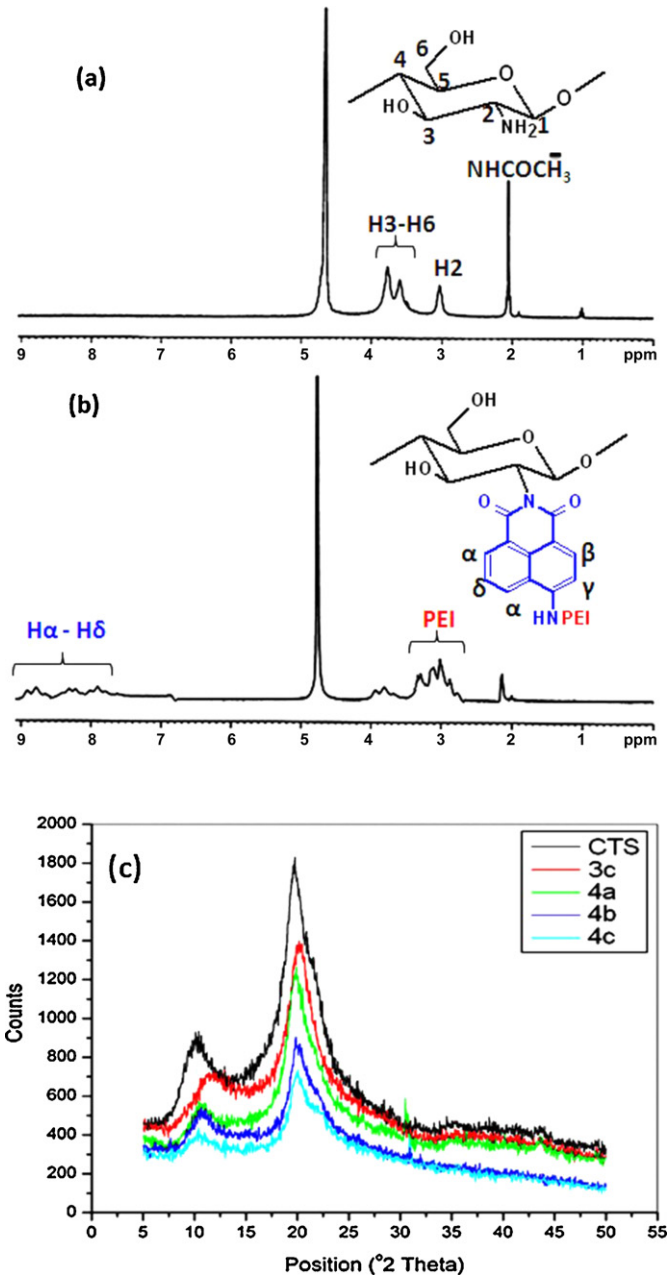


Fig. 2. ^{1}H NMR spectra of chitosan (a), compound **4c** (b) and X-ray diffraction patterns of chitosan and its derivatives.

37.29%, 6.70% and 7.09%, respectively. We prepared compound **3** by reacting chitosan with 4-bromo naphthalic anhydride with three different mol ratios of 1:0.5; 1:0.25 and 1:0.125 (CTS: compound **2**). The solubility of compound **3** markedly decreased at higher mol

Table 1
Elemental analysis (%) and the degree of substitution (DS) of chitosan derivatives.

Sample	C%	H%	N%	DS
CTS	37.29	6.70	7.09	–
3a	45.82	6.85	6.12	18.5
3b	42.37	6.78	6.51	10.4
3c	40.02	6.73	6.79	5.3
4a	41.58	6.89	6.86	8.5[a]
4b	42.13	6.99	6.98	18.2[a]
4c	44.12	7.15	7.24	37.6[a]

[a] DS value of compound **4** is calculated based on compound **3c**.

ratio 1:0.5 with degree of substitution (DS) 18.5% because, the number of primary amine groups of chitosan decreased at higher degree of substitution by the bromo naphthalimide groups. It was also found that the solubility of compound **3** improved with decreasing the DS value (data not shown). The C, H and N contents of CTS markedly increased after grafting of PEI to compound **3** (Table 1). The solubility of compound **4** also markedly increased with increasing the DS value because the number of primary amine groups significantly increased after grafting of PEI with compound **3**. We obtained less soluble product when, we used lower amount of PEI (5%). But, we got soluble product when excess of PEI (30%) was used. This phenomenon can be explained by the fact that when, lower amount of PEI was used, the free primary amine groups of compound **3c** took part in competitive substitution reaction with the primary amine groups of PEI and could substitute the bromine group of compound **3c** to form cross-linked product. But, when large excess amount (30%) of PEI was used, due to smaller molecular size of PEI molecule compared to that of compound **3c**, PEI molecule preferentially reacted with compound **3c** to form the soluble product compound **4c** by the substitution reaction. Similar results were obtained in our previous study (Sarkar & Kundu, 2012).

Therefore, the C, H and N analysis strongly suggests the grafting of PEI into CTS backbone through naphthalimide moiety.

The X-ray diffraction of chitosan, compounds **3c**, **4a**, **4b** and **4c** is shown in Fig. 2c. Chitosan shows two different peaks at $2\theta = 10^\circ$ and $2\theta = 20^\circ$. The peak at 10° was assigned to crystal form I and the strong peak at 20° was assigned to form II (Sarkar, Debnath, & Kundu, 2012). But, for chitosan derivatives, the peak at 10° and 20° significantly decreased. The reason may be attributed to the destruction of the intermolecular hydrogen bonds between the amine groups and hydroxyl groups of chitosan due to the graft copolymerization. These results indicate that the graft copolymerization caused destruction of the ordered crystal structure of the chitosan.

3.2. Fluorescence properties of the copolymer

The fluorescence property of CTS was confirmed by UV–vis spectroscopy and spectrofluorometer. UV absorbance of CTS, compounds **3c** and **4c** is shown in Fig. 3. From Fig. 3a, it is found that chitosan does not show any absorbance within the wavelength 270–400 nm, while compound **3c** shows strong absorbance at 350 nm after incorporation of naphthalimide moiety into chitosan backbone. But, the absorbance maxima of compound **4c** slightly decreased after grafting PEI with chitosan through naphthalimide moiety although the maxima remained at 350 nm.

Emission of radiation from the lowest vibrational level of the excited state to any of the vibrational levels of the ground state is called fluorescence. The fluorescence spectrum, in ideal cases, is therefore a mirror image of the absorption spectrum, but it shifts to longer wavelengths (Stoke's rule) (Zollinger, 2003). Fig. 3b shows the fluorescence excitation and emission (Fig. 3c) spectra of

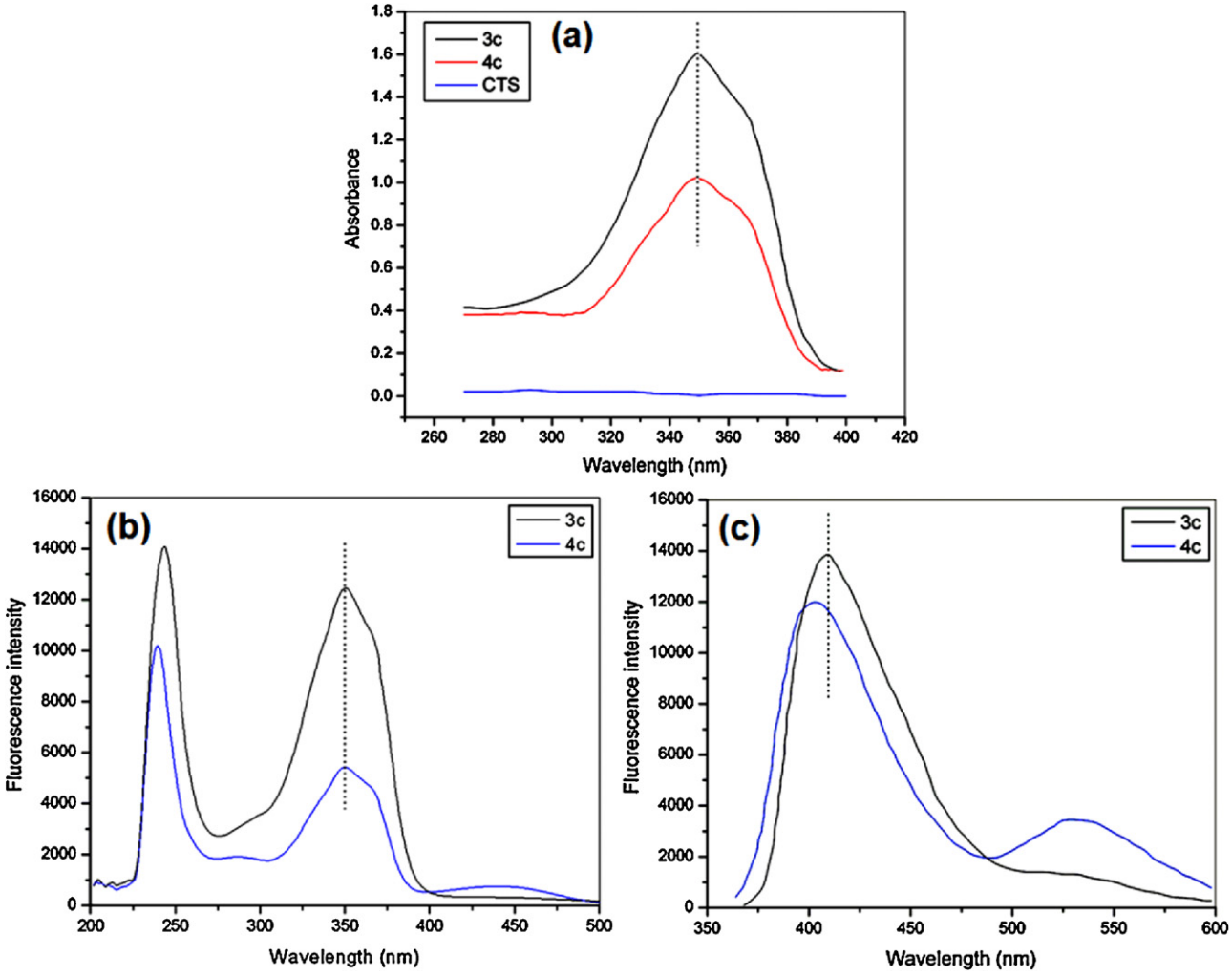


Fig. 3. UV absorbance spectra of chitosan, compound **3c** and compound **4c** (a) and fluorescence excitation (b) and emission (c) spectra of compound **3c** and compound **4c**.

compounds **3c** and **4c**. From Fig. 3b, it is found that both compounds **3c** and **4c** showed the excitation at the same wave length at 350 nm but the compounds exhibited the red shift of 8 nm (λ_{max} = 410 nm for compound **3c**) compared to that of compound **4c** (λ_{max} = 402 nm) (Fig. 3c). This indicates an exciton coupling between the transitions in between the naphthalimide moiety (acceptor) and PEI (donor) by intramolecular photo-induce energy or electron transfer (PET).

3.3. Effect of pH on fluorescence property

The effect of pH on the fluorescence property of fluorescent CTS-*graft*-PEI copolymer (compound **4c**) was carried out at different pH values from pH 7.4 to pH 2.0. Fig. 4 shows the absorbance of compound **4c** at different pH values. As shown in Fig. 4a, the maxima of all absorbance at different pH values remained at 350 nm but the absorbance intensity increased initially with decreasing the pH from 7.2 to 4.0 and then the absorbance sharply decreased with further decreasing the pH from 4.0 to 2.0. When, the pH of the solution decreased, the amine groups (primary, secondary or tertiary) at the surface of the PEI grafted on the fluorescent CTS through naphthalimide moiety were protonated and consequently the sterically crowded structure of compound **4c** converted to less strained structure due to the change in the hybridization of nitrogen atom from sp^3 to sp^2 state. This leads the adjacent nitrogen atom of PEI to undergo better conjugation with the aromatic ring of naphthalimide moiety and consequently increased the absorbance intensity. But, the adjacent nitrogen atom of PEI to naphthalide moiety is protonated at lower pH (pH < 4.0) and consequently the lone pair of electron on the adjacent nitrogen atom are no longer available for conjugation with the aromatic ring of naphthalimide moiety, leading to lowering in the absorbance intensity below the pH 4.0. A possible schematic presentation of the unavailability of the lone pair of electron on the adjacent nitrogen atom is shown in Fig. 4d. The fluorescence spectra showed the similar result with fluorescence emission at 410 nm (excited at 350 nm) (Fig. 4b).

3.4. Effect of temperature on fluorescence property

The effect of temperature on fluorescence property of compound **4c** is also evaluated at different temperatures from 20 °C to 40 °C. Fig. 4c shows the fluorescence spectra of compound **4c** in sodium acetate buffer at pH 5.5 at different temperatures. It is found from Fig. 4c that the maximum fluorescence intensity at 410 nm decreased with increasing the temperature from 20 °C to 40 °C. This fact could be described in terms of both photophysical and photochemical dye properties and polymer chain relaxation processes (Lai, Guan, & Su, 2011).

In the photophysical and photochemical properties of dye, the fluorescence intensity of compound **4c** is mainly controlled by

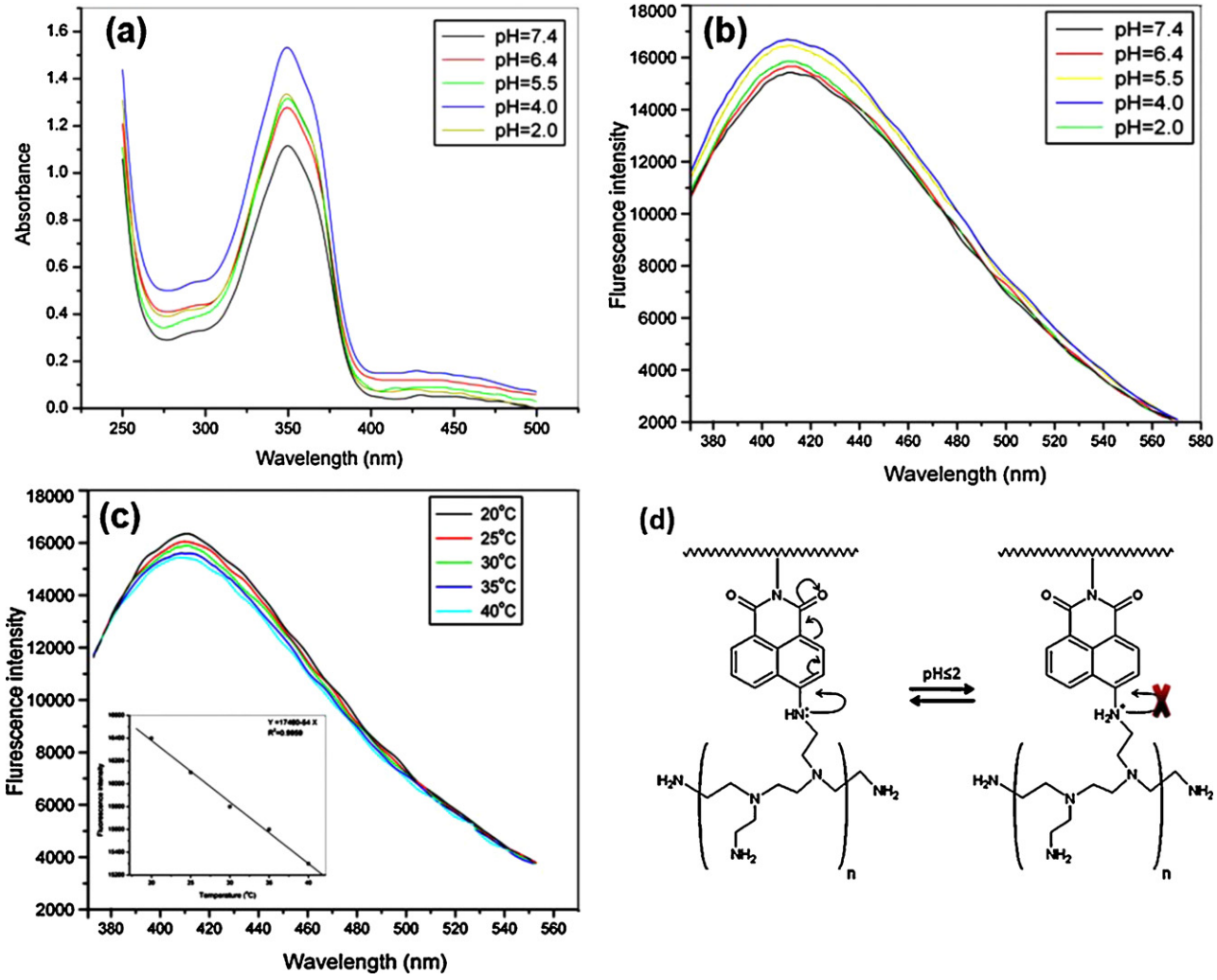


Fig. 4. Effect of pH of the solution on UV absorption spectra (a) and fluorescence spectra (b) of compound **4c**. Effect of temperature on fluorescence spectra (c) of compound **4c** and the linear change of fluorescence intensity with temperature (inset). The possible mechanism for reduce fluorescence intensity at pH ≤ 2 (d).

a radiationless temperature dependent process. The fluorescence quantum yield (Φ_{FL}) of a dye molecule in a homogeneous medium under steady-state conditions and in the absence of either added quenchers or a photochemical process can be described by a general and theoretical equation:

$$\Phi_{FL} = \frac{k_{FL}}{k_{FL} + k_{IC} + k_{ST} + k_{DM}[FL] + k_{MT}^{3}[FL]^{2}}$$

where the rate constants, k, are defined for: fluorescence emission (k_{FL}); radiationless internal conversion (k_{IC}); intersystem crossing (k_{ST}); quenching by collisional and Forster mechanisms (k_{DM}); and triplet–triplet annihilation (k_{MT}), resulting in fluorescence quenching (Talhavini & Atvars, 1998).

The fluorescence intensity of dye molecule can also be influenced by polymer relaxation. In a previous study (Dibbern-Brunelli, de Oliveira, & Atvars, 1995) it was found that the photobleaching yield of fluorescein dye increased with the increase in the mobility of the polymer chains and consequently the destruction of hydrogen bonds in this process could change both the photophysical and photochemical processes of the molecules in electronically excited state.

It is also found that fluorescent chitosan-*graft*-PEI copolymer (compound **4c**) shows excellent linear relationship between the fluorescence intensity and the temperature. From Fig. 4c (inset), it is observed that the fluorescence intensity of compound **4c** linearly decreased with increasing temperature by following linear regression equation, $Y = 17{,}460 - 54X$ (Y = fluorescence intensity and X = temperature) with a correlation coefficient of 0.9959. Therefore, fluorescent CTS-*graft*-PEI copolymer can be used in biomedical field as biological marker.

3.5. Complexation of the copolymer with pDNA

The complex formation between two oppositely charged polyelectrolyte partners can be characterized by the electrophoretic retardation bands in agarose gel. Fig. 5a and b shows the gel retardation results of chitosan/pDNA and fluorescent chitosan-*graft*-PEI (compounds **4a**, **4b** and **4c**)/pDNA complexes at various N/P ratios. It is found that free DNA shows two distinct fluorescent bands, corresponding to the supercoiled and circular forms of the plasmid. When pDNA is complexed with CTS or fluorescent chitosan-*graft*-PEI copolymers at low N/P ratio, a fraction of plasmid is still free to migrate into the gel. Another fraction of plasmid DNA

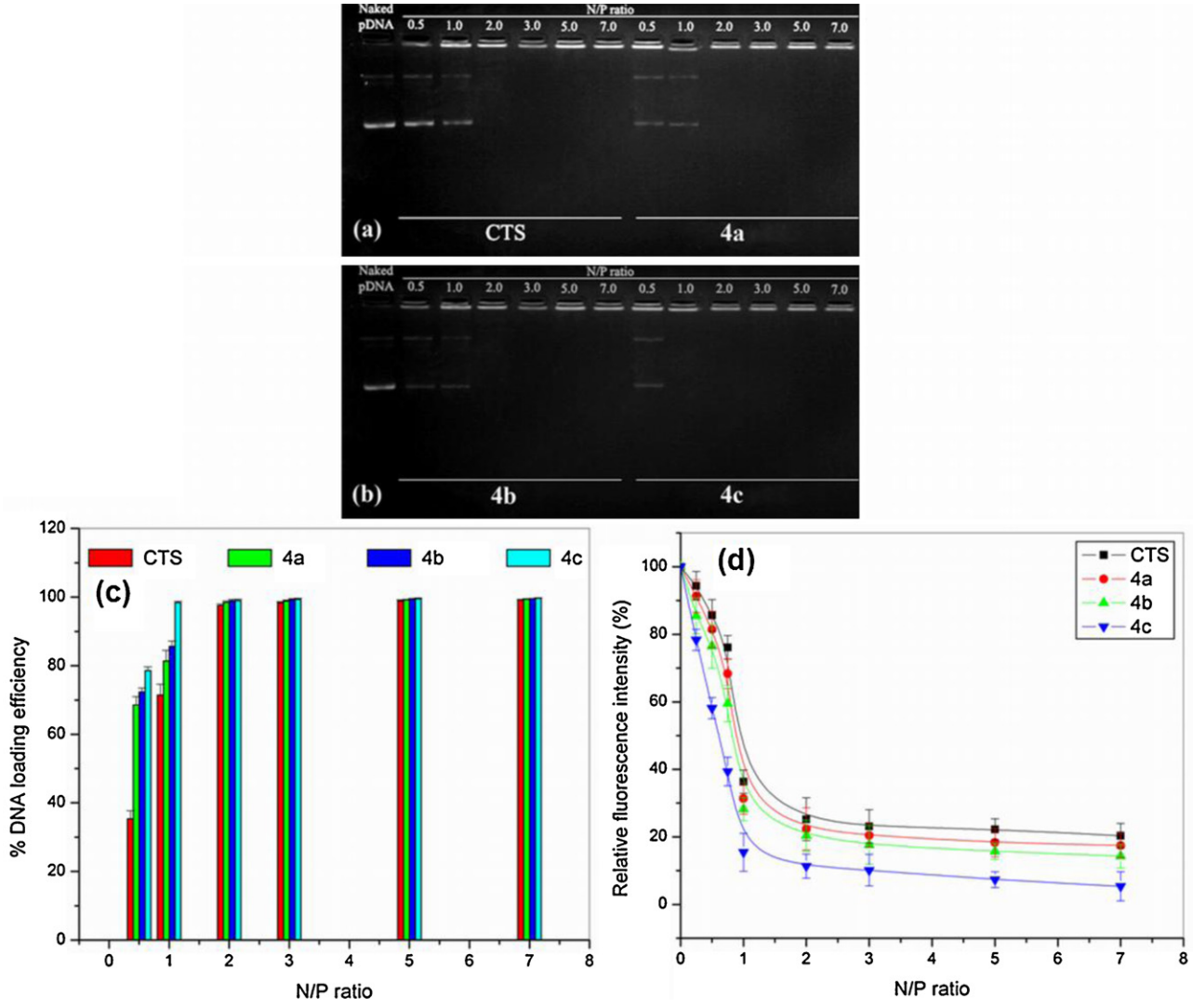


Fig. 5. Agarose gel electrophoresis of chitosan/pDNA complex and compound **4a**/pDNA complex (a) and compound **4b**/pDNA complex and compound **4c**/pDNA complex (b). DNA loading efficiency into CTS and CTS-*graft*-PEI copolymer at different N/P ratios (c). Ethidium bromide assay of CTS and CTS-*graft*-PEI copolymer at different N/P ratios (d).

is trapped in the lane, indicating the formation of complex with larger size than pDNA or less negatively charged than free pDNA. When, pDNA forms complex with CTS and fluorescent chitosan-*graft*-PEI copolymers at N/P ratios of 2 (Fig. 5a, CTS, Lane-4) and 1 (Fig. 5b, compound **4c**, Lane-9), respectively, no DNA migrates into the gel. This indicates that all the pDNA are complexed with the polymer at the respective N/P ratios. From Fig. 5a and b, it is found that compound **4c** is the most effective to complex with pDNA than CTS as well as among the other compounds **4** (compounds **4a** and **4b**). Fig. 5c also shows the similar results where almost all DNA were complexed by compound **4c** at N/P ratio 1.0 whereas, CTS, compound **4a** and compound **4b** condensed all DNA at N/P ratio 2.0. This fact may be explained by the fact that compound **4c** has gained higher positive charge density than chitosan and compounds **4a** and **4b** due to higher substitution degree that increases the primary amine groups at the outer surface of the compound **4c** and consequently the complexation capability of the copolymer increased with the negatively charged pDNA. Therefore, fluorescent CTS-*graft*-PEI copolymer can be effectively used as drug as well as gene carrier vehicle.

3.6. Ethidium bromide displacement assay for complex formation

The binding affinity of polycation with DNA can be determined by ethidium bromide displacement assay. Ethidium bromide (EtBr) intercalates between the base pairs of the DNA double helix, yielding a highly fluorescent DNA/EtBr complex (Dragan et al., 2009). But, the intercalated EtBr is expelled from the DNA/EtBr complex upon addition of polycation due to competitive binding of polycation and EtBr with DNA. As a result, the fluorescence intensity of DNA/EtBr complex decreases. Fig. 5d summarizes the binding affinity of chitosan and chitosan-*graft*-PEI copolymers (compound **4**) with pDNA at different N/P ratios. As shown in Fig. 5d, a rapid displacement of EtBr resulted upon addition of compound **4c** to the DNA/EtBr complex and reached a sharp saturation point around at N/P ratio 1.0. Whereas, the less-pronounced decay of fluorescence was observed for CTS only and reached the saturation point around at N/P ratio 2.0. This observation may be explained by the fact that the cationic charge of the copolymer increased after incorporation the PEI molecule onto the chitosan backbone and resulted in the rapid displacement of EtBr from DNA/EtBr complex due to increased binding affinity of the copolymer with negatively charged DNA. From Fig. 5d, it is also found the EtBr displacement capability of the copolymer increased with increasing the degree of substitution of PEI in the copolymer.

3.7. In vitro cytotoxicity assay

Chitosan is already known as low-toxic and biocompatible polymer. On the other hand, polyethyleneimine is hydrophilic polymer with a high density of primary amine groups. But, PEI has severe toxicity (Sarkar & Kundu, 2012). Therefore, a new derivative of chitosan and PEI should be carefully checked before it is used as biomaterials. The cytotoxicity of CTS, fluorescent CTS-*graft*-PEI copolymers and PEI (25 kDa) on HeLa cells was evaluated by MTT assay at various concentrations of the polymers. As shown in Fig. 6a, fluorescent CTS-*graft*-PEI copolymers exhibit much lower cytotoxicity compared with that of 25 kDa PEI. On the other hand, the cytotoxicity of fluorescent CTS-*graft*-PEI copolymers increases gradually with increasing the concentration. However, the cytotoxicity of PEI increases drastically with increasing concentration and the cell viability becomes below 15% when the concentration reached 100 μg/mL. From Fig. 6a, it is found that chitosan does not show cytotoxicity against HeLa cells in 48 h culture but the toxicity of the copolymer slightly increases with increasing the degree of substitution in the copolymer. It is reported that the cytotoxicity

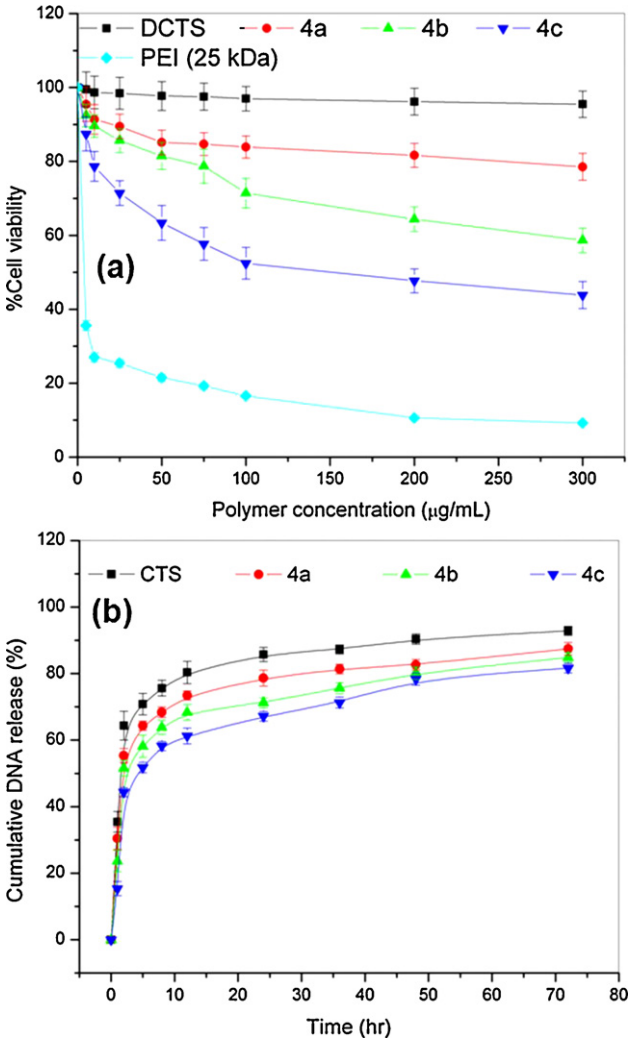


Fig. 6. In vitro cytotoxicity of CTS, compounds **4a**, **4b**, **4c** and PEI (25 kDa) on HeLa cell line (a). In vitro DNA release study from CTS, compound **4a**, **4b** and **4c**/DNA complexes at pH 7.4 (b).

of cationic polymers is probably caused by the interactions with the plasma membrane or interactions with negatively charged cell components and proteins (Fischer, Li, Ahlemeyer, Krieglstein, & Kissel, 2003). When, PEI molecules were grafted to chitosan, the charge density of resulted copolymers increases due to increase in the number of primary amine groups with an increase in degree of substitution. The cell viability remains above 40% and 50% for compound **4c** and compounds **4a** and **4b** copolymer, respectively when the concentration of the polymer reaches 300 μg/mL. These findings demonstrate that fluorescent CTS-*graft*-PEI copolymers are much less toxic compared to PEI. Therefore, fluorescent CTS-*graft*-PEI copolymer can be safely used as the drug as well as a gene carrier.

3.8. In vitro DNA release study

The cumulative release profiles of DNA from fluorescent CTS-*graft*-PEI copolymer/pDNA complex at N/P ratio 2.0 and pH 7.4 were investigated as a function of time at 37 ± 0.5 °C for 72 h. The amount of released DNA was measured by UV spectrophotometer at 260 nm. In Fig. 6b, it is found that the amount of released DNA is increased with time and almost 80% DNA was released from CTS/DNA complex after 12 h, whereas the cumulative releases of DNA from the copolymer decreased with an increase in the

degree of substitution of PEI on CTS. But, almost 80% DNA were released from all copolymer/DNA complexes after 48 h and then remained almost constant. At higher substitution degree of PEI in the copolymer, the number of primary amino groups in the copolymer increases. As a result, the electrostatic interaction between the positively charged polymers and negatively charge pDNA also increases, leading to lowering of the release of DNA from the complex of the copolymer/DNA at a higher degree of substitution of PEI on CTS.

4. Conclusions

In this paper, a novel low toxic fluorescent chitosan-*graft*-PEI copolymer was prepared by grafting of polyethyleneimine with chitosan through naphthalimide moiety. The complexation capability of the copolymer with DNA increased with an increase in the degree of substitution of PEI in the copolymer and compound **4c** showed better DNA complexation ability at lower N/P ratio (N/P = 1.0) compared to that of chitosan (N/P = 2.0). Although, polyethyleneimine is toxic polymer, the toxicity of copolymer was significantly low after incorporation of PEI on chitosan even at higher substituted copolymer (compound **4c**). In vitro release study showed that the cumulative release of DNA from the copolymer/DNA complex slightly reduced compared to that of chitosan due to an increment in charge density in the copolymer. Therefore, this novel chitosan-*graft*-PEI copolymer can be used safely as biological marker in biomedical field and biomacromolecule in future for drug and gene carrier.

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