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Development of mucoadhesive thiolated chitosan nanoparticles for biomedical applications

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ABSTRACT

The main objective of this work was to develop nanoparticles (NPs) of a mucoadhesive polymer based on chitosan for biomedical applications. Here, we developed thiolated chitosan (TCS) using thioglycolic acid (TGA) and chitosan in the presence of 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as catalyst. The prepared TCS was characterized using FT-IR and the degree of thiol substitution was found out by Ellman's method. The TCS nanoparticles (TCS-NPs) were developed using ionic cross-linking reaction with pentasodium tripolyphosphate (TPP). The prepared TCS-NPs were characterized by DLS, AFM, FT-IR, TG/DTA, etc. *In vitro* cytocompatibility and cell uptake studies were also carried out. These studies suggest that the prepared NPs show less toxicity towards normal and cancer cells and they are easily taken up by both the normal and cancer cells. So the prepared TCS-NPs could be used for drug and gene delivery applications.

1. Introduction

Development of bioadhesive/mucoadhesive controlled release systems has been the subject of many studies in recent years. It is widely accepted that limiting the clearance by increasing the viscosity of a drug formulation and also prolonging the contact time through mucoadhesive interactions will result in an increased bioavailability of the drug or whatever therapeutics (Margit, Constantia, & Bernkop-Schnürch, 2003). Polymeric enhancers/mucoadhesive polymers would be a promising candidate for these kinds of applications. They can act as permeation enhancers and thereby they find application in the oral delivery of protein drugs (Lichen, Jieying, Chunbai, Liming, & Cui, 2009). The mucoadhesion, or the attachment of a natural or synthetic polymer to a biological substrate is a practical method of drug immobilization or localization and it is an important aspect of controlled drug delivery (Wu et al., 2009). While the subject of mucoadhesion is not new, there has been increased interest in recent years in using mucoadhesive polymers for drug delivery (Ranabir, Lila, & Sontosh, 2009). Substantial effort has recently been focused on placing a drug or a formulation in a particular region of the body for extended periods of time. This is needed not only for targeting of drugs but also for better control of systemic drug delivery (Ranabir et al., 2009) and the drugs that are absorbed through the mucosal lining of tissues can enter directly into the blood stream and not be inactivated by enzymatic degradation in the gastrointestinal tract (Ranabir et al., 2009). In earlier studies, various natural and synthetic polymers were explored as mucoadhesive excipients (Wu et al., 2009). The mucoadhesive properties of these polymers or the mechanism of mucoadhesion can be explained on the basis of formation of non-covalent bonds such as hydrogen bonds and ionic interactions between the mucoadhesive polymer and the mucus layer (Wu et al., 2009). Several polymeric bioadhesive drug delivery systems have been fabricated and studied in the past. Acrylic-based hydrogels is an example of bioadhesive synthetic polymers which are used for these kinds of applications (Ranabir et al., 2009).

Chitin is a biopolymer, used for various biomedical applications (Jayakumar, Nair, & Tamura, 2009). Chitosan, a biopolymer obtained by the alkaline deacetylation of chitin, is used in the current work. This is a natural polysaccharide of β-(1,4)-linked 2-amino-2-deoxy-D-glucopyranose. Because of its biocompatibility and biodegradation properties (Bernkop-Schnürch, Schwarz, & Steininger, 1999; Jayakumar, Chennazhi, et al., 2010; Jayakumar, Deepthy, Manzoor, Nair, & Tamura, 2010; Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Nair, Tokura, et al., 2010; Jayakumar, Reis, & Mano, 2007), it has been used in a variety of biomedical applications. A number of clinical studies using chitosan have been reported including its use as cell scaffolds in tissue engineering, nerve regeneration tubes, cartilage regeneration (Freier, Koh, Kazazian, & Shoichet, 2005; Khor and Lim, 2003; VandeVord

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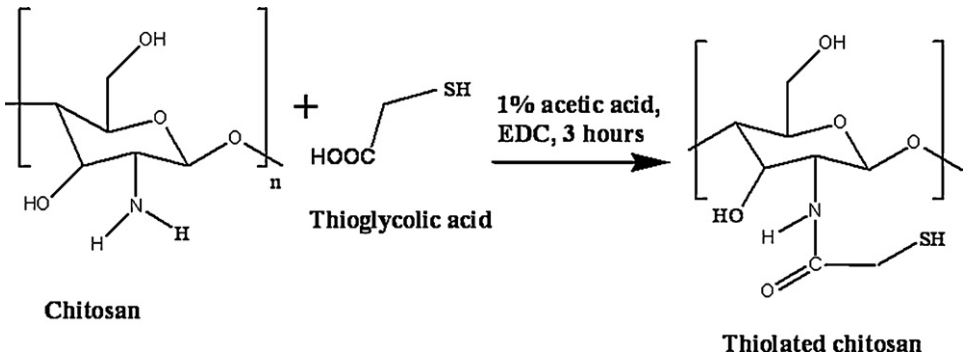

Fig. 1. Reaction scheme for the synthesis of TCS from chitosan using TGA.

et al., 2002) and also in drug delivery applications (Anitha et al., 2009; Dev, Binulal, et al., 2010; Dev, Jithin, et al., 2010; Jayakumar, Chennazhi, et al., 2010; Jayakumar, Deepthy, et al., 2010; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Nair, Tokura, et al., 2010; Jayakumar, Nwe, et al., 2007; Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, Reis, & Mano, 2006; Mathew et al., 2010). Furthermore, it has been used extensively as a biomaterial, owing to its immunostimulatory activities (Mwale et al., 2005), anticoagulant properties, antimicrobial and antifungal action (Gorbach et al., 1994; Rabea, Badawy, Stevens, Smagghe, & Steubaut, 2003) and for its action as a promoter of wound healing in the field of surgery (Madhumathi et al., 2010; Sudheesh Kumar et al., 2010; Wang, Du, Fan, Liu, & Hu, 2005) and in a number of pharmaceutical preparations, primarily for the purpose of controlled drug delivery (Felt, Buri, & Gurny, 1998; Khan, Peh, & Chng, 2000) such as, mucosal (Freier et al., 2005; Gorbach et al., 1994; Mwale et al., 2005), buccal (Rabea et al., 2003), and ocular (Wang et al., 2005) delivery of drugs. NPs based on chitin and chitosan are developed and used for different biomedical applications (Anitha et al., 2009; Jayakumar, Prabaharan, Nair, & Tamura, 2010).

TCS was investigated in this study for biomedical applications. Literatures show that the mucoadhesive properties of TCS are strongly improved in comparison to unmodified chitosan (Khan et al., 2000). When compared with other modified chitosan materials, TCS has numerous advantageous features such as significantly improved mucoadhesive and permeation enhancing properties (Kast & Bernkop-Schnürch, 2001). And these polymers possess hydrophilic properties due to the presence of thiol groups on their side chains (Ronny, Brigitta, Adolf, & Andreas Bernkop, 2008), it is relatively safe and non-toxic (Ronny et al., 2008), its cationic polyelectrolyte character provides strong electrostatic interaction with negatively charged molecules like DNA or drug (Kast and Bernkop-Schnürch, 2001), it displays enzyme inhibitory properties (Ronny et al., 2008). The mucoadhesive property of TCS permits a sustained interaction between the macromolecule being delivered and the membrane epithelia, promoting more efficient uptake properties (Ronny et al., 2008) and it has the ability to open intercellular tight junctions facilitating transport into the cell also via the basolateral membrane (Ronny et al., 2008). Because of these favorable properties TCS have been used for drug and gene delivery (Jayakumar, Chennazhi, et al., 2010; Jayakumar, Deepthy, et al., 2010; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Nair, Tokura, et al., 2010).

In the case of thiolated polymers, the actual mechanism of permeation enhancement can be explained based on the electrostatic interaction between positively charged thiolated polymer and negatively charged sites in the tight junctions, which will lead to drug transport via transiently opened tight junctions. These polymers can tightly adhere to the intestinal mucus layer for a prolonged time through covalent bonding with mucin glycoproteins via thiol–disulfide exchange reactions, hence providing a steeper drug concentration gradient at the absorption sites and exerting an additional permeation enhancing effect (Bernkop-Schnürch, Hornof, & Guggi, 2004). Also polymers with thiol groups, so-called "thiomers" display in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH values. Recently, it has been shown that polymers with thiol groups provide much higher adhesive properties than those polymers generally considered to be mucoadhesive (Bernkop-Schnürch et al., 1999). The enhancement of mucoadhesion can be explained by the formation of covalent bonds between the polymer and the mucus layer, which are stronger than non-covalent bonds (Bernkop-Schnürch et al., 1999).

In this work TCS was developed from chitosan with the help of TGA. The prepared TCS was characterized by FT-IR and degree of thiol substitution was found out using Ellman's protocol. TCS-NPs were prepared by ionic cross-linking reaction. Characterization of the same was done using different characterization techniques. As a preliminary work before going in to biomedical applications, cell compatibility studies of the prepared NPs were tested by MTT assay in different cell lines (both normal and cancer). In addition cell uptake studies of TCS-NPs were also conducted by rhodamine conjugation of TCS-NPs.

2. Experimental

2.1. Materials

Chitosan (molecular weight 100–150 kDa and degree of deacetylation 80%) was purchased from Koyo Chemical Co., Ltd., Japan, TPP, dialysis tubings (molecular weight cut-off 12 kDa), Triton X-100, EDC and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma Aldrich, TGA from Merck, breast cancer cell line (MCF-7), fibroblast cell line (L929) and mouse embryonic fibroblast (NIH3T3) for cell culture experiments were purchased from NCCS, Pune and all other chemicals used are of analytical grade.

2.2. Synthesis of TCS

TCS was synthesized based on the reported literatures (Bernkop-Schnürch et al., 1999, 2004; Lichen et al., 2009; Margit et al., 2003). The reaction scheme for TCS synthesis is shown in Fig. 1. The coupling reaction of chitosan with TGA was mediated by a carbodiimide, EDC. Briefly 500 mg of chitosan was dissolved in 50 ml 1% acetic acid. Thereafter EDC dissolved in 1 ml demineralized water was added to a final concentration of 125 mM. After proper mixing of these two, 500 mg TGA was added and the pH of the medium was adjusted to 5. The synthesis was performed at a pH 5.0, in order to avoid the formation of disulfide bonds between

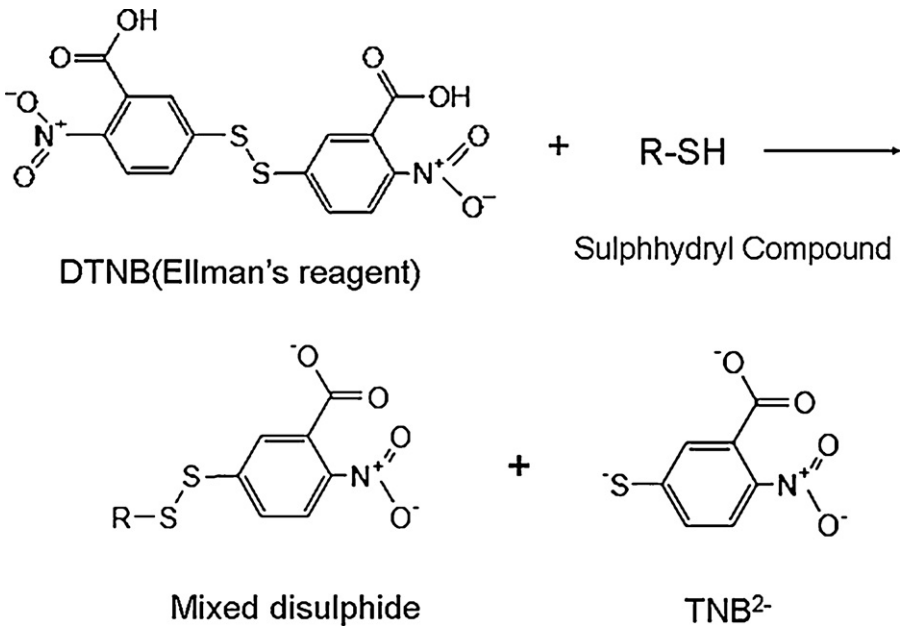

Fig. 2. Reaction scheme showing Ellman's method.

the polymer chains by oxidation during the coupling reaction. At that pH, the amount of the reactive form for the oxidation of thiol groups (thiolate anions) is low and the formation of disulfide bonds can be minimized (Bernkop-Schnürch et al., 2004). Then the reaction mixture was incubated for 4 h in dark at room temperature under constant stirring. In order to isolate the TCS from the reaction mixture, the polymer solutions were dialyzed in tubings of cellulose membrane with a molecular weight cut-off of 12 kDa for 3 days in dark against 5 mM HCl, then twice against the same medium but containing 1% NaCl to reduce the ionic interactions between the cationic polymer and the anionic sulfhydryl groups. For maintaining the pH of the medium as 4, the samples were again dialyzed against 5 mM HCl for 2 days. Control samples were made in the same way without adding TGA. During dialysis, the sample containers were kept in dark at 4 °C for avoiding the oxidation of sulfhydryl groups. After dialysis, the samples and controls were lyophilized by drying the frozen aqueous polymer. The lyophilized product was used for further studies.

2.3. Quantification of thiol group content (determination of degree of thiol substitution/determination of free sulfhydryl groups) by Ellman's method

The degrees of thiol group substitution in these modified polymers were determined spectrophotometrically using Ellman's reagent as described in literature (Margit et al., 2003). Ellman's method is based on the reaction of Ellman's reagent with sulfhydryl bearing compounds. Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid or DTNB) is a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryl groups. Consequently, Ellman's reagent is very useful as a sulfhydryl assay reagent because of its specificity for –SH groups at neutral pH, DTNB reacts with free sulfhydryl groups to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) as shown in Fig. 2. The target of DTNB in this reaction is the conjugate base ($R–S^-$) of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several factors such as the reaction pH, the pK_a of the sulfhydryl and steric and electrostatic effects. TNB is the 'colored' species produced in this reaction and has a high molar extinction coefficient in the visible range. Sulfhydryl groups can be estimated in a sample way, by comparing with a standard curve composed of known concentrations of a sulfhydryl-containing compound such as TGA.

Briefly, a 2 mg/ml solution of polymer was prepared in millipore water. Then 250 μl aliquots were added to 250 μl of 0.5 M phosphate buffer (pH 8.0) and to 500 μl of Ellman's reagent (0.3 mg/ml of DTNB in 0.5 mol/l phosphate buffer pH 8.0). The reaction was allowed to proceed for 2 h at room temperature and the absorbance was measured at a wavelength of 420 nm. Control samples were elaborated with non-modified chitosan. The amount of thiol moieties was calculated from the corresponding standard curve elaborated between 0.25 and 2 mM of thioglycolic acid solution in water (Bernkop-Schnürch, Kast, & Guggi, 2003).

2.4. Characterization of TCS

FT-IR spectra of chitosan and TCS were recorded on Perkin-Elmer Spectrum RX1 Fourier transforms infrared spectrophotometer using KBr method. The new amide bond formation and thiol group substitution in TCS can be confirmed by FT-IR, based on the presence of the characteristic peaks of newly formed amide bond and thiol groups.

2.5. Preparation of TCS-NPs

TCS-NPs were obtained as a result of ionic cross-linking reaction with a cross-linker, TPP (Anitha et al., 2009; Devika & Varsha, 2006). The TCS prepared was dissolved in water and kept for stirring. NPs were obtained as result of the addition of TPP solution to the aqueous solution of polymer; TCS to TPP weight ratio used is 3:1. As a result of ionic cross-linking, a turbid solution was obtained, which was kept for half an hour stirring. The resultant TCS-NPs were separated from the stable suspension by centrifugation at 13,000 rpm for 1 h at 10 °C. Then the pellet was redispersed in water and lyophilized, the lyophilized sample was used for further characterization and studies.

2.6. Characterization of TCS-NPs

2.6.1. Measurement of particle size by DLS measurements

The mean size and size distribution of the prepared NPs were determined by DLS measurements using DLS-ZP/Particle Sizer Nicomp™ 380 ZLS.

2.6.2. Stability studies—determination of zeta potential

The surface charge and thereby the stability of the TSC-NP system was determined by zeta potential measurements using a DLS-ZP/Particle Sizer Nicomp™ 380 ZLS.

2.6.3. Measurement of particle size and morphology by AFM

The size and morphological characteristics of the prepared TCS-NPs were further confirmed by AFM (JEOL JSPM-5200). For AFM, the NP suspension was successively diluted in millipore water and it was spread on an aluminium disc and dried at room temperature. The resulting sample was used for AFM imaging.

2.6.4. FT-IR

In order to confirm the formation of TCS from TGA, i.e. to study the chemical interaction between each of these constituents, FT-IR spectra was taken by Perkin-Elmer Spectrum RX1 Fourier transform infrared spectrophotometer using KBr method. The freeze dried TCS-NPs were mixed with KBr and pressed in to a pellet and kept for scanning.

2.6.5. Thermal studies

The thermal behavior of the chitosan, TCS and TCS-NPs were studied using TG/DTA analysis. TG/DTA analysis was performed with SII TG/DTA 6200 EXSTAR between 20 and 500 °C with a 10 °C/min heating rate under nitrogen atmosphere. This study was used to study the moisture content in each of them and their thermal degradation behavior.

2.7. Cell culture

L929, NIH3T3 and MCF-7 cell lines were used in this study. The cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in CO_2 incubator at 37 °C with 5% CO_2. After reaching confluency, the cells were detached from the flask with Trypsin-EDTA. The cell suspension was centrifuged at 3000 rpm for 3 min and then re-suspended in the growth medium for further studies. After third passage, the cells were split and used for experiments.

2.8. In vitro cytocompatibility studies

In vitro cytocompatibility of the prepared TCS-NP was done by MTT assay in two normal (L929 and NIH3T3) cell lines and one cancer cell line (MCF-7). MTT assay is a colorimetric test based on the selective ability of viable cells to reduce MTT in to purple colored formazan crystals. These formazan crystals have an absorbance maxima at 570 nm. The formazan crystals produced were dissolved in a solubilisation buffer and absorbance was taken at 570 nm. The absorbance obtained is directly proportional to the number of viable cells. Based on that, a graph was plotted i.e. the percentage cell viability versus TCS-NP concentration in mg/ml.

MTT assay was done for a range of concentrations from 0.2 to 2 mg/ml with normal cell cultured wells without any particles as negative control. Then the particles along with cells were incubated for 48 h. Then MTT solution (10%) was added and kept for 4 h of incubation and then solubilisation buffer was added and again incubated for 1 h. After complete dissolvation of MTT, the absorbance was measured at a wavelength of 570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment.

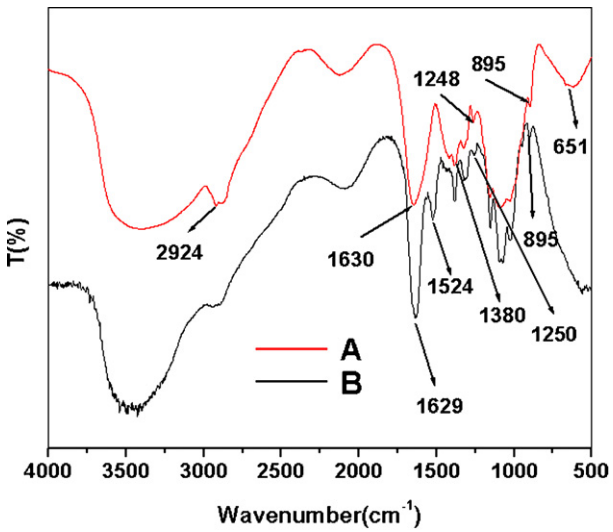

Fig. 3. FT-IR spectra of chitosan (A) and TCS (B).

2.9. In vitro cell uptake studies

In vitro cell uptake studies of TCS-NP was done by rhodamine 123 conjugation of TCS-NPs. Cell uptake studies were done in two normal (L929 and NIH3T3) and one cancer cells (MCF-7). For this, acid etched cover slips were placed in 24 well plates and were seeded with a density of 10,000 cells per cover slip and incubated for 24 h for the cells to attach. After 24 h incubation, the media was removed and the wells were carefully washed with PBS buffer. Then the rhodamine 123 conjugated TCS-NPs at a concentration of 1 mg/ml was added along with the media to each of the wells and incubated for 24 h. There after the sample containing media was removed and the cover slips were dried and processed for imaging using fluorescent microscopy. The processing involved washing the cover slips with PBS and thereafter fixing the cells in 5% paraformaldehyde (PFA) followed by a final PBS wash. The cover slips were air dried and mounted on to glass slides with DPX as the mountant medium. The slides were then viewed under the fluorescence microscope. The experiment was performed in triplicates.

Table 1
Cell viability (%) values of NIH3T3, L929 and MCF-7 for the different concentrations of TCS-NPs.

Concentration (mg/ml)	Cell viability (%)		
	L929	NIH3T3	MCF-7
0	100	100	100
0.0	98.86587	98.85827	99
0.4	98.77119	98.89136	98.8
0.6	98.6685	98.75415	99
0.8	98.47955	98.25249	98.7
1	98.34721	98.84385	98.6
1.2	98.03924	98.73887	98.8
1.4	98.56	98.92284	98.9
1.6	98.74197	98.81462	98.7
1.8	98.53151	98.96678	98.6
2	98.75591	99.11628	98.9

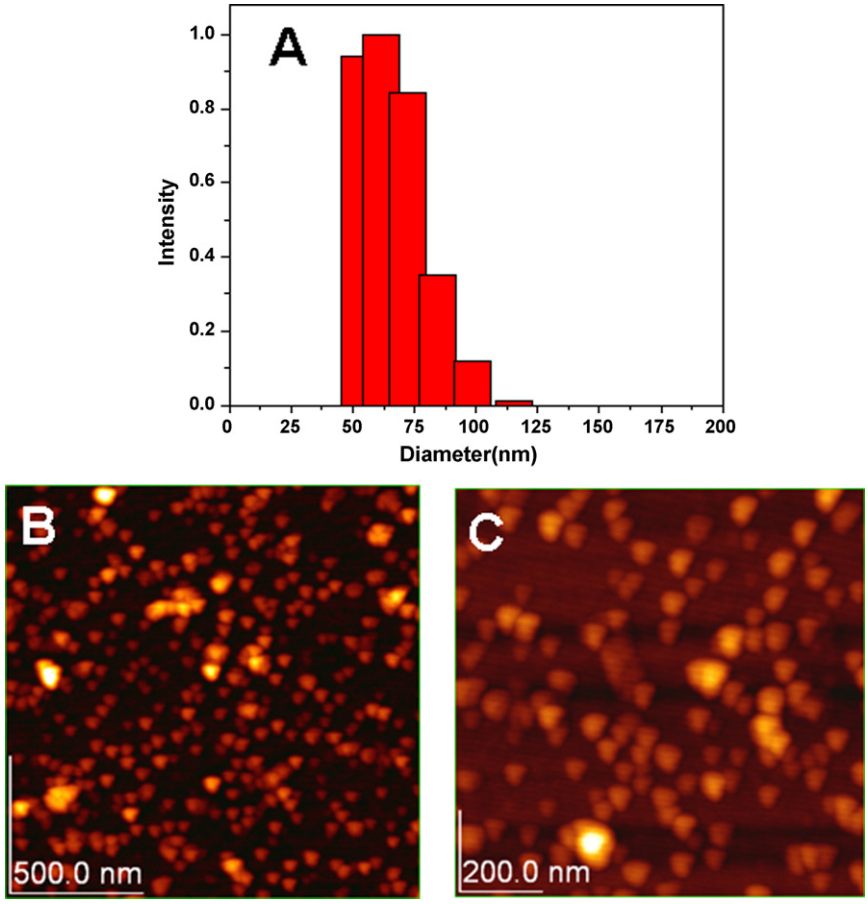

Fig. 4. (A) DLS, (B) and (C) AFM images of TCP-NPs .

3. Results and discussion

3.1. Preparation and characterization of TCS

TCS was synthesized using TGA as the thiolating moiety and the characterization was done using FT-IR. TCS was obtained as a result of the formation of amide bond between NH_2 groups of chitosan and carboxyl groups of TGA via EDC catalyzed reaction and the new amide bond formation between chitosan and TGA can be confirmed by FT-IR. The degree of thiol substitution was found out using Ellman's protocol and it was found to be 60%.

Fig. 3 represents the combined FT-IR spectra of chitosan and TCS. In the spectrum of chitosan, the characteristic absorption peaks are at about 3410 cm^{-1} (ν_{O-H} and ν_{N-H}), 2924 cm^{-1} (ν_{C-H}), 1623, 1513 cm^{-1} (δ_{N-H}), 1088 cm^{-1} (ν_{C-N}), 651 cm^{-1} (δ_{NH_2}), 1380 cm^{-1} (δ_{C-H}), 1248 cm^{-1} (δ_{O-H}), 1153 cm^{-1} (δ_{C-O-C}), 651 cm^{-1} (due to amino groups) and 895 cm^{-1} (epimeric β-δ_{C-H} of cyclic pyranosyl rings) could be easily observed. In TCS all the peaks in chitosan were present, except the peaks corresponding to amino (NH_2) groups, since amino groups in chitosan were reacted with carboxyl groups of TGA resulting in an amide bond, so the additional peaks of this newly formed amide bond and peaks of thiol groups (from the TGA

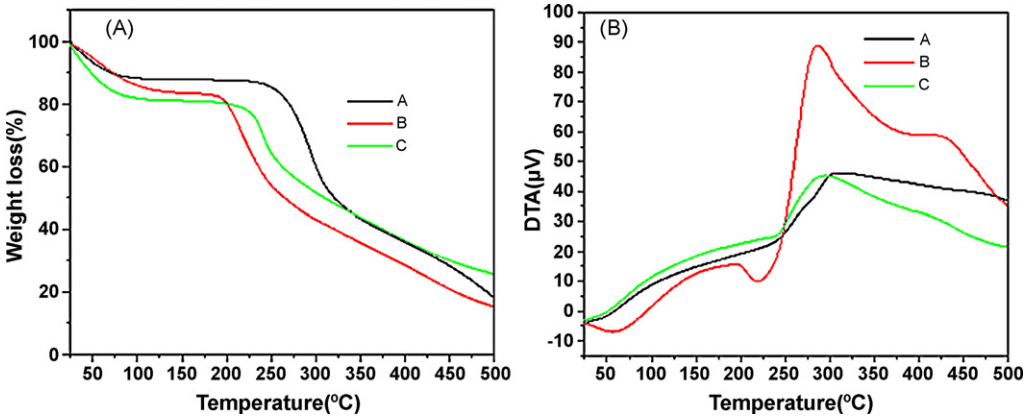

Fig. 5. (A) and (B) TGA and DTA of (A) chitosan, (B) TCS and (C) TCS-NPs.

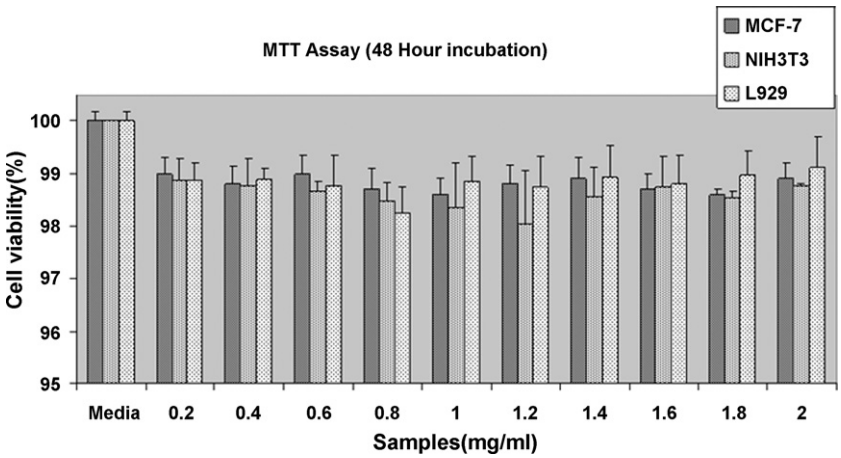

Fig. 6. MTT assay of TCS-NPs towards L929, NIH3T3 and MCF-7.

moiety) was observed. The wave numbers corresponding to these are 1524 (amide II band), 1629 (amide I band) and 1250 cm^{-1} peak corresponds to thiol groups (Bernkop-Schnürch et al., 1999, 2003, 2004).

3.2. Preparation and characterization of TCS-NPs

TCS-NPs were obtained as a result of the ionic cross-linking reaction between the positively charged protonated amine groups of TCS and negatively charged phosphate groups of TPP. By adjusting the concentration of the precursors and stirring speed, we can control the degree of cross-linking and thus the particle size.

3.2.1. Particle size analysis using DLS measurements

Using DLS measurements, the size distributions for the TCS-NPs were obtained. Fig. 4A shows the particle size distribution for TCS-NPs. The average diameter of the TCS-NPs was found to be 50–100 nm.

3.2.2. Particle size and morphology studies

The size of the prepared NPs were also confirmed by AFM. Fig. 4B and C represents the AFM images of TCS-NPs at different magnifications. These particles showed a size range of 80–100 nm in AFM. From the AFM images, it is clear that the prepared TCS-NPs possess spherical morphology.

3.2.3. Stability of the TCS-NP system by zeta potential measurements

Surface charge and thereby the stability of the prepared NP systems was determined by zeta potential measurements. The zeta potential value for the TCS-NP system was found to be +43.69 mV. This value indicates that the prepared system is extremely stable and the TCS-NPs possess positive surface charge. This net positive surface charge can be explained by the presence of more protonated amino groups in TCS-NPs surface.

3.2.4. FT-IR studies

FT-IR spectra of TCS and TCS-NPs were taken, the characteristic peaks of TCS are at 3410 cm^{-1} (ν_{O-H} and ν_{N-H}), 2924 cm^{-1} (ν_{C-H}), 1623, 1513 cm^{-1} (δ_{N-H}), 1088 cm^{-1} (ν_{C-N}), 651 cm^{-1} (δ_{NH_2}), 1380 cm^{-1} (δ_{C-H}), 1248 cm^{-1} (δ_{O-H}), 1153 cm^{-1} (δ_{C-O-C}), 651 cm^{-1} (due to amino groups) and 895 cm^{-1} (epimeric β-δ_{C-H}, of cyclic pyranosyl rings), 1524 (amide II band), 1629 (amide I band) and 1250 cm^{-1} peak corresponds to thiol groups.

In the NPs spectrum, i.e. the TPP cross-linked TCS, all the peaks of TCS were present, but the intensity of most of them reduced especially the 1637 peak, this can be explained by the ionic cross-linking reaction of TPP with N–H groups of TCS that is ultimately leads to NP formation (Anitha et al., 2009; Devika and Varsha, 2006).

3.2.5. TG/DT analysis

3.2.5.1. TGA. The thermal degradation behavior of chitosan, TCS and TCS-NPs are shown in Fig. 5A and B. Fig. 5A represents the TGA of chitosan, TCS and TCS-NPs. From the figure it is clear that chitosan started to degrade at 250 °C and has a broad degradation temperature range with a high char yield at 550 °C. The degradation mechanism is very complex including dehydration, deacetylation and chain scission. The first-stage degradation from 200 to 250 °C was attributed to the loss of water (Trong-Ming, Chung-Yang, & Wen-Yen, 2002). For TCS, the degradation starts at a lower temperature compared to chitosan. This can be explained by the fact that the chain scission in the case of TCS is easier because of the presence of easily breakable side chains (due to thiolation –NH–CO–CH_2–SH), where as in the case of TPP cross-linked TCS i.e. in TCS-NPs degradation is much difficult than TCS, this can be explained by the TPP cross-linking, this makes the thermal degradation difficult (Khalid, Agnely, Yagoubi, Grossiord, & Couarraze, 2002).

The DTA curves for chitosan, TCS and TCS-NPs were shown in Fig. 5B. In all the three curves, an endothermic peak was observed around a temperature of 200–275 °C and exothermic peaks observed between 270 and 310 °C these are characteristics of chitosan (Khalid et al., 2002).

3.3. In vitro cytocompatibility studies by MTT assay

In vitro cytocompatibility of the prepared TCS-NP was determined by MTT assay. The test was done for a range of concentrations from 0.2 to 2 mg/ml, with normal tissue culture medium without any NPs as negative control. Fig. 6 shows a plot that describes the percentage cell viability versus concentration of the NPs in mg/ml. Table 1 gives the cell viability values in percentage for the respective cell lines with respect to the concentration of TCS-NPs.

From the plot it was clear that, there is no significant difference in percentage cell viability in any of the concentrations of the NPs samples studied. Similarly, we also compared the level of toxicity of NPs with the negative control, which is the normal tissue culture medium. It was evident from the figure that compared to the negative control almost 98% cells are viable in all the different concentrations of TC-NPs. These results indicated that the prepared TCS-NPs are less toxic to breast cancer cells (MCF-7) and normal cells (L929 and NIH3T3) and this is an essential requirement for a material to be used for biomedical applications.

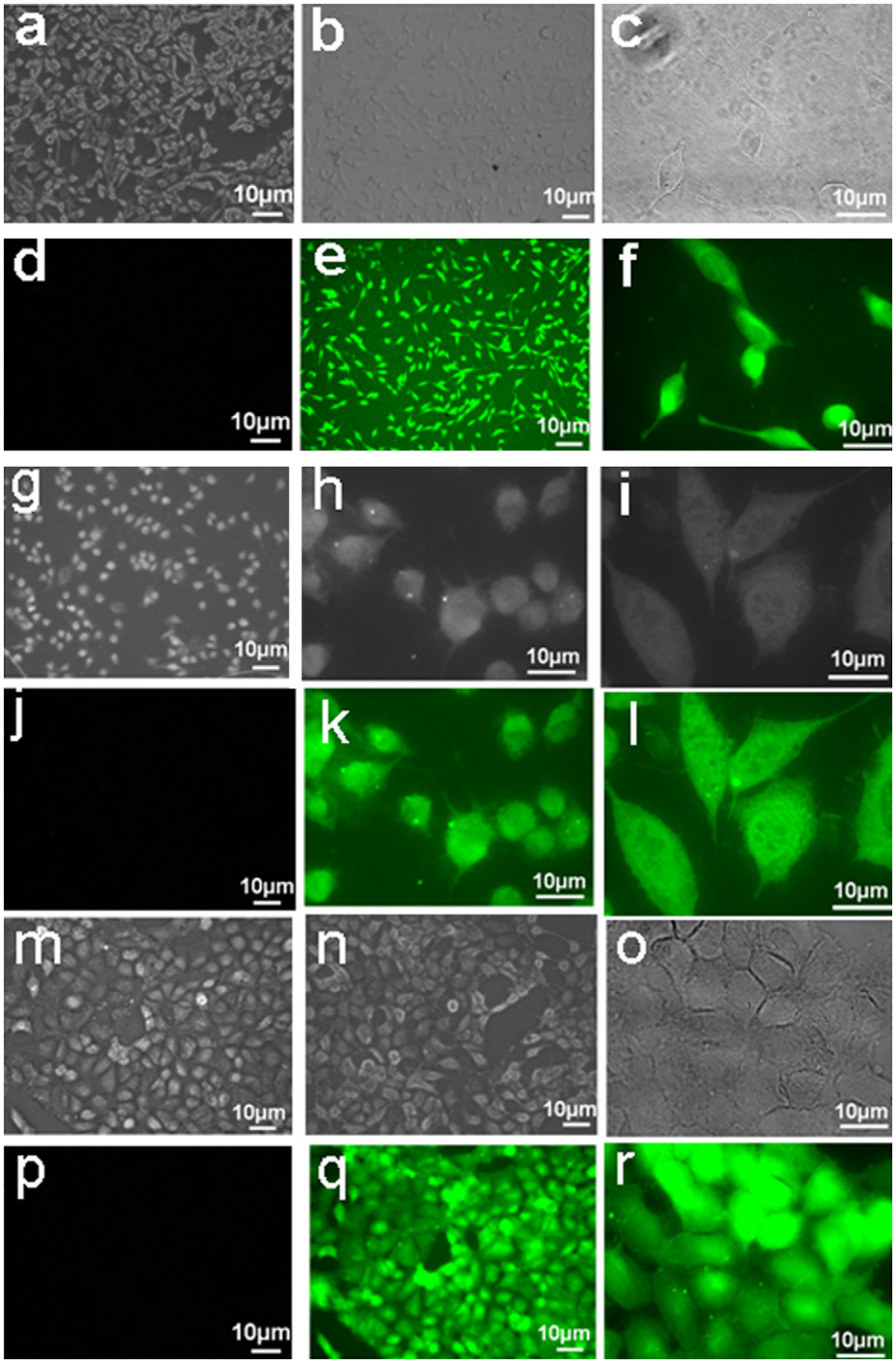

Fig. 7. Cell uptake studies of rhodamine conjugated TCS-NPs in L929 (a–c, bright field and d–f, fluorescent images), NIH3T3 (g–i, bright field and j–l, fluorescent images) and MCF-7 (m–o, bright field and p–r, fluorescent images) cells.

3.4. In vitro cell uptake studies using fluorescent imaging

In vitro cellular uptake studies of rhodamine 123 conjugated TCS-NPs were studied by visualizing the bright green fluorescence coming from the rhodamine dye using fluorescence microscopy. Fig. 7 shows the microscopic images of fluorescence study. Control cells without any NPs showed no fluorescence. Cells incubated with rhodamine conjugated TCS-NPs showed green fluorescence confirming the internalization of the TCS-NPs inside the cells. Also, it is evident from the fluorescence images (Fig. 7) that even after incubation with particles; the cells have retained its normal morphology which further demonstrates the biocompatibility of the TCS-NP system.

4. Conclusions

A mucoadhesive polymer, TCS was synthesized and characterized. Degree of thiol substitution was found out using Ellman's protocol and it was found to be 60%. The prepared TCS-NPs were

characterized using DLS, AFM, FT-IR and TG/DTA studies. The prepared NPs possess a size range of 80–110 nm and spherical morphology. Stability studies were done by zeta potential measurements and the system was found to be extremely stable and the NPs possess positive surface charge. *In vitro* cytocompatibility of the prepared NPs were studied using MTT assay towards normal and cancer cell lines. These results showed that the prepared NPs are less toxic (almost 98% cells are viable in all the different concentrations TCS-NPs samples). So the prepared TCS-NPs can be used as an efficient biomaterial for biomedical applications such as mucoadhesive drug delivery, gene delivery, etc.

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