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Drug loaded thermoresponsive and cytocompatible chitosan based hydrogel as a potential wound dressing

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ABSTRACT

There is a demand for a wound dressing which can be removed from the application site easily without causing any pain or discomfort. A material removable by manipulating the temperature (e.g. moisten with ice cold water), from that point of view, seems to have considerable potential. Here we report a new formulation consisting of thiolated chitosan with poly(N-isopropyl acrylamide) loaded with ciprofloxacin. The thermoresponsive material was cytocompatible and was found to modulate the release of the incorporated ciprofloxacin in a sustained fashion reflecting its suitability to protect a wound for a prolonged period. The film exhibited adequate mechanical strength and was removable from a substrate (e.g. tissue culture plate) by lowering the temperature. The combination of thiolated chitosan with poly(N-isopropyl acrylamide) and ciprofloxacin showed antibacterial properties to the virulent bacteria E coli supporting its potential as a wound dressing.

1. Introduction

Wound dressings and devices form an important segment of the medical and pharmaceutical wound care market world wide (Boateng, Matthews, Stevens, & Eccleston, 2008). Wound dressings are used to protect the site of injury from further insult, contamination and infection that may impede healing. The ideal wound dressing would facilitate and accelerate wound healing and their easy removal without causing any pain to the patient. Wound exudate is a key component in all the stages of wound healing, irrigating the wound continuously and keeping it moist (Gray & White, 2004). The maintenance of a moist wound bed is widely accepted as the most ideal environment for effective wound healing (Eaglstein, Davis, Mehle, & Mertz, 1988). However, in chronic wounds, there is excessive amounts of exudates present which can lead to complications. Pathogenic bacteria can be detrimental to the healing process. Inadequate measures to manage infected wounds can lead to cellulites, bacteraemia and septicemia, which can be fatal. Therefore a number of factors need to be controlled and managed effectively for the fabrication of an ideal wound dressing.

Traditional dressings include cotton wool, natural or synthetic bandages and gauzes. Gauze and saline are useful for the initial stages of wound healing for absorbing blood and exudates, cleansing and debridement. These dressings are dry and do not provide a moist environment. In addition gauze dressings tend to become more adherent to wounds as fluid production diminishes and are painful to remove, thus causing patient discomfort. Gauze dressings also provide little occlusion and allow evaporation of moisture resulting in a dehydrated wound bed. The history of traditional dressings and problems associated with their use has been reviewed by Jones (Jones, 2006).

Modern dressings have been developed as an improvement upon the traditional wound healing agents. Their essential characteristic is to retain and create a moist environment around the wound to facilitate wound healing. The modern dressings are mainly classified according to the materials from which they are produced including hydrocolloids, alginates and hydrogels, and generally occur in the form of gels, thin films and foam sheets. A detailed review of the common wound management dressings, their merits, demerits and emerging technologies for achieving improved wound healing is done by Boateng et al. (2008).

Chitin and chitosan are widely used as wound dressings and haemostatic agents (Jayakumar, Chennazhi, et al., 2010). Chitosan, [β-(1-4)-2-amino-2-deoxy-D-glucose] is the 70–80% N-deacetylated derivative of chitin which is one of the most abundant polysaccharides in nature. The exoskeleton of marine arthropods like shrimp, lobster and crabs are the main industrial sources of chitosan (Felt, Buri, & Gurny, 1998). The N-acetyl glucosamine (NAG) present in chitin and chitosan is a major component of dermal tissue which is essential for repair of scar tissue (Singh & Ray, 2000). Since chitosan is biodegradable, non-toxic and biocompatible it has received considerable attention in various fields

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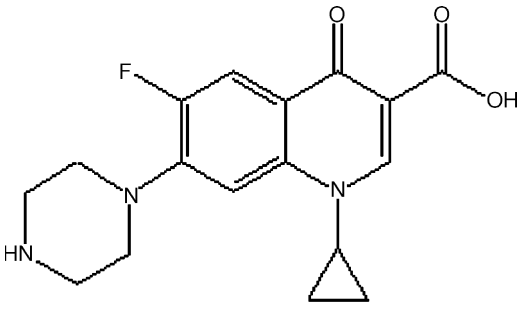
of pharmaceutical technology (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Deepthy, Manzoora, Nair, & Tamura, 2010; Takeuchi, Yamamoto, & Kawashima, 2001). Due to the easy availability of free amino groups in chitosan, it carries a positive charge and is amenable to chemical modifications. In a review Jayakumar, Prabaharan, et al. (2010) have detailed the applications of carboxymethyl derivatives of chitin and chitosan, as drug delivery systems, in wound healing, as anti-microbial agents and in tissue engineering. Chitosan has been shown to possess mucoadhesive properties owing to the interaction between positively charged chitosan with the glycoproteins of the negatively charged mucosal surfaces (Kas, 1997; Peppas & Mikos, 1990). To further enhance the mucoadhesive properties of chitosan various derivatives such as trimethylated chitosan, mono-N-carboxymethyl chitosan, N-sulfochitosan and chitosan-EDTA conjugates were developed (Baumann & Faust, 2001; Bernkop-Schnürch & Krajicek, 1998; Thanou, Florea, Langemeyer, Verhoef, & Junginger, 2000; Thanou, Nihot, Jansen, Verhoef, & Junginger, 2001). There are reports stating that polymers with thiol groups provide enhanced adhesive properties than many polymers generally considered to be mucoadhesive. Different thiolated chitosan derivatives have been reported in the literature (Bernkop-Schnürch & Hopf, 2001; Bernkop-Schnürch, Brandt, & Clausen, 1999; Bernkop-Schnürch, Hornof, & Zoidl, 2003; Hornof, Kast, & Bernkop-Schnürch, 2003; Kast & Schnürch, 2001). The thiolated polymers are believed to interact with cysteine rich subdomains of mucus glycoproteins via disulfide exchange reactions (Kast & Schnürch, 2001). The strong cohesive properties of thiolated chitosans make them highly suitable for controlled drug release dosage forms.

N-isopropylacrylamide (NIPAAm) monomer has frequently been used for assigning thermo responsiveness to polymer matrices (Shivkumar, Mozetic, & Paradossi, 2009). Since poly(NIPAAm) expels its liquid contents at a temperature near that of the human body, it has been investigated for possible applications in controlled drug delivery (Yan & Tsujii, 2005).

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) is a quinolone antibiotic drug indicated for the treatment of skin and skin structure infections and also for other indications like respiratory infections urinary tract infections, the gastrointestinal surgery, typhoid fever, gonorrhea, and septicemia. Ciprofloxacin act by inhibiting the bacterial enzymes DNA gyrase. Its empirical formula is C17H18FN3O3 and its chemical structure is shown below.



CIPROFLOXACIN

We reasoned that a combination of thiolated chitosan and poly(NIPAAm) containing a wide spectrum antibacterial drug like ciprofloxacin could be an interesting matrix for evaluating as a smart, drug eluting mucoadhesive gel having the synergistic properties of natural and synthetic entities. Moreover, the thin film of this composite wound dressing having thermoresponsive property, swells when wiped with cold water and can be removed very easily without causing any trauma to the patient's skin.

2. Experimental

2.1. Materials

Chitosan (from crab shells, 75% deacetylated, viscosity average molecular weight 195 kDa), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), mercaptopropionic acid, and N-isopropyl acrylamide (NIPAAm) are procured from Sigma–Aldrich, Bangalore, India. Ciprofloxacin was from Fluka, USA. α′,α′-Azobisisobutyronitrile (AIBN) and other analytical grade chemicals were from Spectrochem Pvt Ltd., Mumbai, India. Dulbecco's Minimum Essential Medium (DMEM) was obtained from Sigma Chemical Co., St. Louis, MO, USA. The materials used for the antibacterial studies are purchased from the following sources. Beef infusion, casein hydrolysate and agar were procured from HiMedia Laboratories, Mumbai, India. Starch was procured from Sisco Research Laboratories, Mumbai, India. *Escherichia coli* (ATCC 25922) was obtained from American Type Culture Collection, Rockville, MD, USA. The medium used for testing the antibacterial activity was Muller Hinton Broth/Agar. All other materials were of analytical or equivalent grade.

2.2. Preparation of mercaptopropionic acid modified chitosan (thiol-chitosan)

2% w/v chitosan in 2% aqueous acetic acid was prepared by overnight stirring at room temperature using a mechanical stirrer. 2 ml mercapto propionic acid was first activated with 50 mM EDC and added to the chitosan solution. The pH of the solution was maintained at 5.0. The contents were stirred at room temperature for 6–8 h. The products were dialysed first against 5 mM HCl for 2 days, 5 mM HCl containing 1% NaCl for 1 day and finally twice against 1 mM HCl to remove unreacted mercaptopropionic acid (Atyabi, Moghaddam, Dinarvand, Zohuriaan-Mehr, & Ponchel, 2008). The purified thiolated chitosan solution was lyophilized after freezing at −80 °C for 24 h.

2.3. Determination of thiol group content of mercaptopropionic acid modified chitosan

To determine the degree of substitution of thiol groups on chitosan, iodine titration was performed as done by Atyabi et al. (2008). 10 ml of thiolated chitosan after purification by dialysis is taken in a conical flask and titrated with an aqueous iodine solution (1 mM) using starch indicator, until a permanent light blue colour was obtained.

2.4. Preparation of a blend of thiol-chitosan with poly(NIPAAm)

The lyophilized thiolated chitosan was dissolved in 100 ml distilled water. The above solution was taken in a 3 necked round bottom flask; 8 g NIPAAm monomer was added and stirred until the solution become homogeneous. The flask was kept in a water bath and nitrogen was bubbled through it. 100 mg AIBN dissolved in a small quantity of acetone was added and the temperature of the water bath was increased to 60 °C. The reaction was allowed to take place for 3 h, under constant stirring. The contents were poured into a disposable polystyrene mold and cured at 45–50 °C for 48 h. The film formed was neutralized with 2% aqueous sodium hydroxide solution, extensively washed with distilled water to remove unreacted reactants and residual sodium hydroxide and refrigerated.

The virgin chitosan, thiolated chitosan and the blend of thiolated chitosan with poly(NIPAAm) were coded as CH-0, CH-T and CH-T-PNi, respectively.

3. Characterization

3.1. Fourier transform infra red (FTIR) spectroscopy

The FTIR spectra were recorded in range 600–4000 cm^{-1} using a Diamond ATR accessory in Nicolet 5700 FTIR Spectrometer, Madison, USA. The analysis was done based on American Society for Testing and Materials (ASTM) international standards (ASTM E 573-01).

3.2. Thermal analysis

Differential scanning calorimetry (DSC 2920, TA Instruments Inc., USA) was used to determine the lower critical solution temperature of the CH-T-PNi blend films. The material was pre-equilibrated in distilled water for 1 h, 10–15 mg of the sample was taken in a hermetic aluminum pan and heated from 0 to 50 °C and cooled back to 0 °C at the rate of 3 °C min^{-1} in an atmosphere of nitrogen. The procedure used for the DSC analysis was base on (ASTM E-537-07).

Thermogravimetric analysis (TGA) was done (SDT 2960, Simultaneous DTA-TGA, TA Instruments Inc., USA) to determine the thermal stability and compositional analysis of the CH-T-PNi blend samples. 10–12 mg of the materials were taken in a platinum cup and heated under nitrogen atmosphere at a heating rate of 10 °C min^{-1} from room temperature to 600 °C. The test method was derived from (ASTM E-1131-03).

3.3. Contact angle

The CH-T-PNi blend films were conditioned in double distilled water for 24 h and the water contact angles were measured using an OCA 15 Plus Video based contact angle measuring device (Dara Physics, Germany) as per the instruction manual. The water drops were dispersed on wet film surface and the contact angle on both sides were taken. The contact angle of the same drop was measured repeatedly after every 2 min to observe the change in contact angle resulting from intermolecular interaction between the polymer surface and water drop. A minimum of 6 readings were taken and averaged.

3.4. Swelling percentage

Square film samples of 100 mm^2 size (of known mass) were immersed in phosphate buffer of pH 7.4 for known intervals of time. The strips were removed and carefully blotted between filter paper to remove excess fluid and weighed. Swelling index = $[W/W_0 \times 100]$, where W is the (final weight − initial weight), and W_0 is the Initial weight. A minimum of 6 readings were taken and averaged.

3.5. Mechanical properties

Tensile properties of the CH-0 and CH-T-PNi blend films were determined in the wet state using Universal Testing Machine (Instron 3345, UK) as per ASTM D 882 (2002). The films were kept in distilled water and conditioned in the testing atmosphere, at 23 °C for 24 h. Dumbbell shaped strips of 3-mm width were cut and thickness measured using a micrometer. Full-scale load range of 100 N was applied at a cross head speed of 100 mm/min. Stress at break; percentage elongation and elastic modulus were calculated. The computed values are the mean of 6 repeat measurements.

3.6. Sterilization of the cleaned samples

Thoroughly cleaned and sterilized samples were subjected to ethylene oxide (ETO) sterilization in a 3 M Steri-Vac 8XL Gas sterilizer cum Aerator. The unit is designed to sterilize heat and moisture sensitive devices. Steri-Gas ethylene oxide cartridge 8-170 is used as the ethylene oxide source. The sterilization is done at 55 °C for 1 h at 40–60% humidity with an aeration time of 4 h.

3.7. Surface topography of the blend films

The surface topography of the blend film was studied at 24 °C using an Agilent 5500 Atomic force microscope (Agilent technologies, USA) in the contact mode. Micro-fabricated silicon cantilever tips (PPP - N9832-80002) (Agilent technologies, USA) was used for the measurement at a scan rate of 1 Hz.

3.8. The in vitro cytotoxicity studies

The *in vitro* cytotoxicity test of the CH-T-PNi blend films were carried out by both direct contact assay and test on extract method as per (ISO 10993-5, 1999) The cytotoxicity of the 1 week extracts of the blends were quantitatively assessed further by MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide] assay (Ciapetti, Cenni, Pratelli, & Pizzoferrato, 1993).

3.8.1. Direct contact method

Cleaned & ETO sterilized film samples of surface of area 0.1–0.3 cm^2 were used for the direct contact test. Test samples, negative controls (high density polyethylene) and positive controls (copper) in triplicate were placed on confluent monolayer of L-929 mouse fibroblast cells. After incubation of the cells with test samples at 37 ± 2 °C for 24 ± 1 h, the fibroblast cells were evaluated for general morphology, vacuolization, detachment, cell lysis and degeneration. Cellular responses were examined and scored as 0, 1, 2 and 3 with 0 indicating non-cytotoxic, 1 mildly cytotoxic, 2 moderately cytotoxic and 3 severely cytotoxic.

3.8.2. Test on extract method

The test on extract was done to prove that the degradation products of the blends if any are not cytotoxic. The extract was prepared by incubating cleaned, dried and ETO sterilized films of area 6 cm^2 and thickness <0.5 mm, with 1 ml culture medium (DMEM) containing FBS (foetal bovine serum) at 37 °C for 1 week. Different dilutions of the extracts with medium, negative control (high density polyethylene) and positive control (copper) in triplicate were placed on sub-confluent monolayer of L929 cells and incubated at 37 ± 2 °C for 24 ± 1 h. The cell culture was then stained with trypan blue and examined microscopically for cellular responses. The dead cells stain deep blue.

3.8.3. MTT assay

MTT assay is a standard colorimetric assay for the quantification of living cells. MTT dissolved at a concentration of 5 mg/ml in sterile PBS (phosphate buffered saline), filtered through a 0.22 μm filter to remove any formazan crystals (MTT metabolic product) and stored at −20 °C was used as the stock solution. 1:10 dilution of the stock solution was prepared as the working solution. 6000 cells in 100 μl tissue culture medium were plated in a 96 well flat bottom tissue culture plate. The plate was then incubated at 37 °C in 5% carbon dioxide overnight. When monolayer was attained, culture medium was removed, rinsed with PBS and 100 μl each of extracts of the film (100, 50 and 25%) and negative control and 100 μl of diluted phenol (positive control), 0.1% (v/v) in tissue culture medium were added to different pre-labeled wells containing cells. Cells with medium alone served as the control. Plates were

again incubated for 24 h at 37 °C in 5% carbondioxide atmosphere. After 24 h, the extracts/medium was removed and 100 μl of MTT working solution was introduced using a multi-channel pipette into each well. Plates were wrapped with aluminum foil and incubated for 8 h as before. After removing the reagent solution and rinsing with PBS, 200 μl of DMSO (dimethylsulfoxide) (100%) was added to each well and incubated for 20 min at 37 °C in a shaker incubator (Orbitek, SCIGENICS BIOTECH) to ensure that the dye (formazan-MTT metabolic product) is completely dissolved. The absorbance of the resulting solution in each well was recorded immediately at 570 nm using automated micro-plate reader (Model UVM 340, ASYS, Austria). Background was subtracted at 670 nm. Reported values are the means of three replicates.

3.9. Ciprofloxacin uptake and its in vitro release by the blend film

Ciprofloxacin is sparingly soluble in water but freely soluble in dilute aqueous alkaline solution. 10 mg/ml ciprofloxacin in 0.05N aqueous NaOH was prepared. The pre-weighed films having a size of 1 cm^2 were dipped in 1 ml of the drug solution and kept overnight. Subsequently films were taken out, rinsed gently with distilled water and dried at 37 °C until the weight was constant.

Each of the drug loaded CH-T-PNi blend films was suspended in 1 ml phosphate buffer (pH 7.4) at 37 °C in an orbital shaker. After definite intervals of time, e.g. 0.25, 0.5, 1, 2, 3, 24, and 48 h, the buffer solution was withdrawn and replaced with fresh buffer. The amount of ciprofloxacin present was estimated spectrophotometrically using a Varian, Cary 100 Bio UV-Visible Spectrophotometer based on the ASTM procedure (ASTM E 169-04). A calibration curve using the absorbance of different concentrations of ciprofloxacin at a wavelength of 274 nm was prepared and the amount of ciprofloxacin released was calculated from the curve. A blank film of the blend without drug was used as the control. Triplicate analyses were done.

To calculate the ciprofloxacin uptake by the blend film, the following procedure was used. The film after 48 h drug release study was digested with 2 ml of 0.5N aqueous acetic acid solution at 37 °C for 24 h and the remaining ciprofloxacin was estimated spectrophotometrically. The values obtained were added to the amount of drug released during the 48 h study to obtain the total drug uptake by the film. A blend film without drug, treated in the same manner was used as a blank.

3.10. Determination of antibacterial activity

Antibacterial activity was studied using the bacterial strain *E. coli* (ATCC 25922). Muller Hinton broth (MH broth)/Agar, media was prepared using 300 ml of beef infusion, 17.5 g casein hydrolysate, 1.5 g starch, 1 g agar and 1000 ml distilled water. The starch was emulsified in a small quantity of cold water and mixed with other constituents and were dissolved by heating gently at 100 °C with agitation. The pH was adjusted to 7.4. Sterile media autoclaved at 121 °C for 20 min was transferred to sterile tissue culture plates (Nunc, Denmark) and maintained at a uniform depth of 4 mm.

Antibacterial activity of the drug loaded films was checked by Minimum Inhibitory Concentration (MIC) method using tube dilution followed by subculturing on Muller Hinton Agar. The drug loaded films after ETO sterilization were placed in 5 ml of Muller Hinton broth for 24 h release of ciprofloxacin. The concentrations were based on the amount of ciprofloxacin release calculated by UV–vis spectrophotometry. One ml of the above solution was taken and doubling dilutions were made in different tubes to get a final volume of 1 ml in each tube. Then a standard inoculum of 10 μl of bacteria (ATCC E coli) having 10^5/colony forming units/ml was inoculated into each tube and incubated for 18 h or overnight with intermittent shaking at 37 °C. The next day turbidity and clarity in each tube were noted, with turbidity indicating growth of the bacteria and clarity denoting inhibition of bacteria by the concentration of released ciprofloxacin. Subcultures were done from each tube by disc diffusion on to Muller Hinton Agar to confirm the viability of bacteria. MIC is the 'lowest concentration' that shows clarity in the tube and on subculturing the organism was completely inhibited. The MIC of ciprofloxacin was also checked by tube dilution method.

Disc diffusion on Agar plate was done by incorporating 10 μl of ciprofloxacin or the released antibiotic on a 6 mm diameter sterile filter paper disc. After overnight incubation at 37 °C the zone of inhibition was measured.

3.11. Statistical analysis of results

Results, expressed as mean ± SD were evaluated by '*t*' test and 'analysis of variance' (ANOVA). Values of $p < 0.05$ were considered statistically significant.

4. Results and discussion

4.1. Preparation of a blend of thiol-chitosan with poly(NIPAAm)

In one of the review papers Jayakumar et al. have summarized the different methods of preparation of sulfated chitin and chitosan and their properties. This review also summarized some of the recent applications of these materials in various fields such as adsorbing metal ions, biomedical, anti-microbial and drug delivery (Jayakumar, Reis, & Mano, 2007; Jayakumar, Nwe, Tokura, & Tamura, 2007). In our study 2-mercaptopropionic acid was reacted with chitosan through the primary amino group at the C2 of the glucosamine subunit for generating thiolated chitosan. The carboxylic acid group of the mercaptopropionic acid reacts with the primary amino group of chitosan mediated by a water soluble carbodiimide. The degree of thiolation was found to be 535 μmol/g chitosan. Studies have indicated that thiolated chitosan has 5–10-fold increase in mucoadhesion in comparison to unmodified chitosan. The ability of the thiolated chitosan to form covalent bonds with the mucus layer is attributed to the enhanced mucoadhesion. Beside stronger mucoadhesiveness, thiolated derivatives display excellent cohesive properties which enable them as stable matrices for controlled drug delivery applications (Bernkop-Schnürch, Hornof et al., 2003; Bernkop-Schnürch, Kast, & Guggi, 2003; Guggi, Krauland, & Bernkop-Schnürch, 2003; Kast, Valenta, Leopold, & Bernkop-Schnürch, 2002).

The CH-T-PNi blends showed good film forming property. The thermoresponsive property of the blends is clear when they are kept on a granite surface. The film undergoes phase transition and changes from transparent to opaque form under wet and dry conditions as depicted in Fig. S1 in supplementary data.

4.2. Fourier transform infra red (FTIR) spectroscopy

In Fig. 1 the characteristic peaks of chitosan (CH-0) due to the OH/–NH_2 stretching, –C=O– stretching and –NH_2 bending are located at 3361, 1638 and 1579 cm^{-1}, respectively. Jayakumar et al. (2007a, 2007b) reported that the peaks at 1254, 1154 and 896 cm^{-1} are due to thiol groups for their chitosan-thiolactic acid conjugate. In our study the thiolation of chitosan in CH-T was confirmed from the prominent band due to –CH_3 group of the mercaptopropionic acid at 1482 cm^{-1} and the strengthened band of the amide II signal at 1579 cm^{-1} suggesting that addition was occurred at the chitosan amine site. In the blend, CH-T-PNi, the –C=O– stretching band is broadened due to the combined absorption due to the –C=O– stretching of the poly(NIPAAm) and chitosan at 1658 and 1640 cm^{-1}, respectively. The already existing amide II band (–NH_2

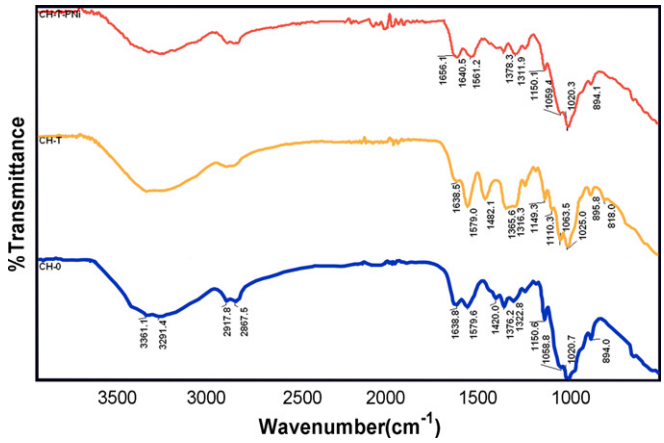


Fig. 1. FTIR Spectra of the virgin chitosan (CH-0), thiolated chitosan (CH-T) and the blend (CH-T-PNi) films.

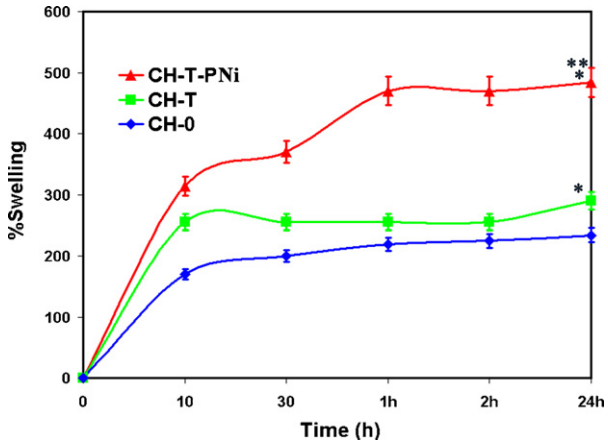


Fig. 3. Swelling percentage of virgin chitosan (CH-0), thiolated chitosan (CH-T) and the blend (CH-T-PNi) films (*$p = 0.005$ and 0.007 for CH-T and CH-T-PNi respectively when compared to CH-0; **$p = 0.0008$ for CH-T-PNi when compared to CH-T).

bending) of chitosan was shifted from 1579 cm^{-1} to lower wavelength 1561 cm^{-1} in the blend which could be due to the formation of hydrogen bond between the amino groups of chitosan and the carbonyl groups of poly(NIPAAm). Ahn, Choi, and Cho (2001) have made similar observation of the shifting of the carbonyl peak to lower frequency in poly acrylic acid–chitosan complex.

4.3. Thermal analysis

DSC scan (Fig. S2, supplementary data) shows the lower critical solution temperature (LCST) of the CH-T-PNi blend. The transition in the cooling curve is marked for the LCST in order to eliminate the irreversible thermal transitions of the sample in the first heat curve.

Poly(NIPAAm) is a typical temperature sensitive hydrogel having a LCST at 32–33 °C. Its phase transition behaviour can be altered by incorporating hydrophilic or hydrophobic polymers in the gel composition. The LCST for the blend is 30.4 °C confirming that the reversible phase transition of poly(NIPAAm) is retained in the blend membrane also. The presence of the poly(NIPAAm) makes the polymer blend an active material forming a switchable surface as depicted in Fig. S1 (supplementary data). In water at its LCST, the blend film undergoes a phase transition and become a swollen polymer. Above the LCST, the polymer collapses to a state having much lower water content. This property change makes the removal of the membrane from the applied surface easier when it is wetted with cold water.

The thermogravimetric analysis (TGA) of the CH-0, CH-T and CH-T-PNi is shown in Fig. 2. The thermal stability of chitosan is marginally decreased as an effect of thiolation. In virgin chitosan, the first decomposition temperature (Ti) was observed at 258 °C and that of CH-T at 231 °C. This is attributed to the thermal destabilization of the chitosan chains due to the dissociation of the strong intra-molecular hydrogen bonding among them as an effect of thiolation. However, on blending with poly(NIPAAm) the thermal stability of chitosan is increased as seen from its elevated Ti at 267 °C. The char residue of the blend decreased at the end of decomposition (T_{end}), from 26% for CH-0 to 18.5% for CH-T-PNi, implying a decreased thermal stability of the blend at elevated temperature. TGA of the blend polymer showed a two stage decomposition corresponding to the thiolated chitosan and the poly(NIPAAm). The thermal decomposition data are compiled in Table S1 (supplementary data). The corresponding DTG of the polymer films clearly confirms the two stage decomposition for the blend in contrast to the single stage decomposition of CH-0 and CH-T. The peak around 292 °C is due to the decomposition of chitosan and the peak around 406 °C is assigned to the degradation of poly(NIPAAm) in the blend.

4.4. Contact angle

Virgin chitosan, CH-0 is hydrophobic in nature having a water contact angle of 103° as shown in Fig. S3 in supplementary data. The CH-T, showed more hydrophilic character than CH-0 as evident from its contact angle value of 95°. On the other hand, the high water contact angle, 113° obtained for the CH-T-PNi blend film indicates that the film surface is highly hydrophobic. How-

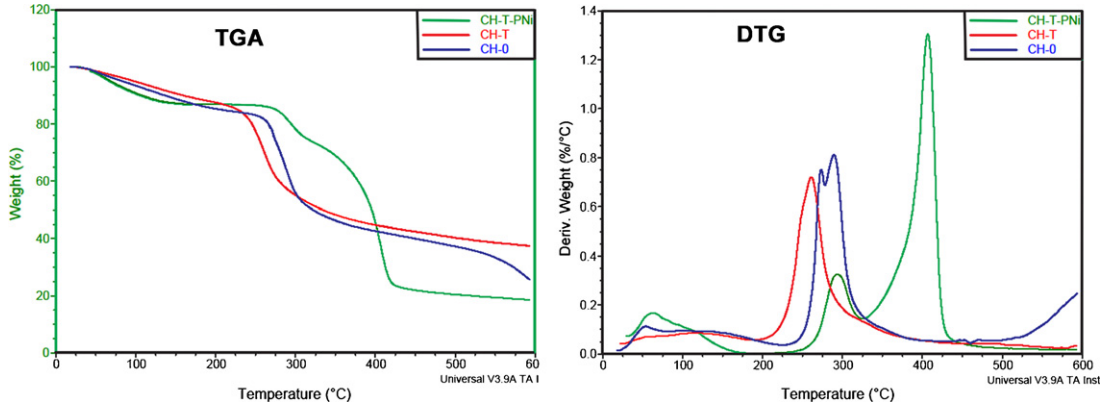


Fig. 2. Thermogravimetric analysis (TGA) and derivative thermogram (DTG) of virgin chitosan (CH-0), thiolated chitosan (CH-T) and the blend (CH-T-PNi) films.

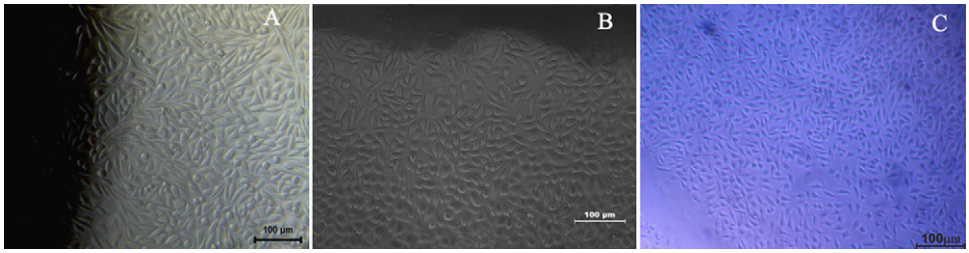


Fig. 4. (A) CH-T-PNi blend film, (B) negative control (high density polyethylene) in contact with L929 mouse fibroblast cells, (C) trypan blue stained image of the 1 week extract (100%) of the CH-T-PNi blend film in contact with L929 mouse fibroblast cells.

ever, a time bound decrease in the contact angle was observed and the angle stabilized at 47°, indicating transformation of the hydrophobic surface to a hydrophilic one. The blend film showed the LCST at 30.4 °C as evident from its DSC scan. On equilibration with water at the experimental temperature of 25 °C, the poly(NIPAAM) moieties in the blend attained a varied conformation acquiring hydrophilicity causing a reduction in its water contact angle.

4.5. Swelling percentage of the blend film

The % swelling of the samples as depicted in Fig. 3 were studied in phosphate buffer at pH 7.4. The average moisture content of the CH-0, CH-T and CH-T-PNi samples calculated from the TGA is 16, 14 and 13%, respectively (Table S1, supplementary data). It has been reported that chitosan is hydrophobic in nature and its swelling index at pH 7.4 is low (Mathews, Guinness, Birney, & Cahill, 2006). On thiolation, chitosan is expected to increase hydrophilicity which is confirmed by our results. However, on formation of a blend of thiolated chitosan with poly(NIPAAm) the hydrophilicity was further increased. This observation can be assigned to the phase change of poly(NIPAAM) from hydrophobic to hydrophilic since the experimental temperature was 25 °C which is below the LCST of poly(NIPAAm). The statistical analysis shows that the swelling percentage of CH-T and CH-T-PNi is significantly different when compared to CH-0. It is also found that when compared to CH-T the swelling percentage of CH-T-PNi is significantly different.

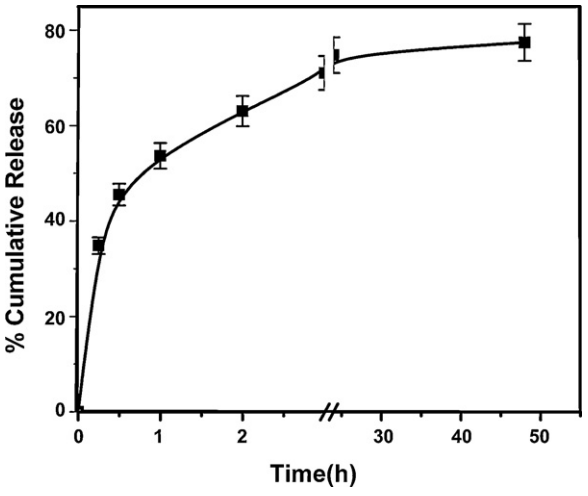


Fig. 5. Cumulative release of ciprofloxacin from the thiolated chitosan poly(NIPAAm) (CH-T-PNi) blend film ($p = 4.9 \times 10^{-10}$ when the drug release at different time intervals are compared to one another).

4.6. Mechanical properties

The mechanical properties of the films are compared in the wet condition as the blend films are projected for wound dressing applications. It is seen from Table 1 that, blending with PNIPAAm resulted in a decrease in the tensile strength of chitosan. CH-0 film exhibited a tensile strength of 51.1 ± 6.8 MPa while the CH-T-PNi film had a value of 3.4 ± 0.6 MPa. The corresponding elongation was 92.3 ± 6.8 and 332 ± 50%, respectively. The modulus value of the CH-0 film also showed a significant decrease on blending with PNIPAAm. The hydrogen bonding with water breaks down intermolecular interaction among the functional groups of chitosan and PNIPAAm. In the case of virgin chitosan, the plasticization effect of water enhances its strength and elongation. Zhang, Li, Gong, Zhao, and Zhang (2002) reported that the mechanical property of the chitosan film is decreased on blending with PEG. Blair, Guthrie, La, and Turkington (1987) reported that when chitosan is blended with PVA, none of the blend membranes was as strong as the chitosan or the poly(vinyl alcohol) membranes. According to him a small amount of PVA in the blend produced a large reduction in strength and elasticity of chitosan. Our observation is also similar to that of Blair. However, it is reported that high elongation and low elastic modulus are ideal for burn dressings (Boateng et al., 2008). Hence, the mechanical parameters obtained for the blend indicate that it meets the requirements for a potential wound dressing material.

4.7. Surface topography of the blend films

The morphology of the CH-T-PNi blend films was observed by AFM. The frictional image (Fig. S4, supplementary data) reveals the non-uniformity of the surface. Two different surfaces of different mechanical properties can be observed. This may be due to the composite nature of the film formed from the blends of CH-T and poly(NIPAAm).

4.8. The in vitro cytotoxicity studies

4.8.1. Direct contact method

The CH-T-PNi blend films were thoroughly cleaned by ultrasonication and ETO sterilized before subjected to cytotoxicity evaluation. Fig. 4A is the cytotoxicity evaluation of the CH-T-PNi blend film by direct contact method. Fig. 4B is the image of the L929 cells in contact with the negative control (high density polyethylene). Normal L929 fibroblast cells are spindle shaped, healthy and viable, with a glistening appearance (Fig. 4B). Non-toxic materi-

Table 1
Mechanical properties of the virgin chitosan (CH-0) and the blend (CH-T-PNi) films.

Sample code	Tensile stress (MPa)	% Strain	Young's modulus (MPa)
CH-0	51.1 ± 6.8	92.3 ± 6.8	71 ± 14
CH-T-PNi	3.4 ± 0.6	332 ± 50	1.4 ± 0.2

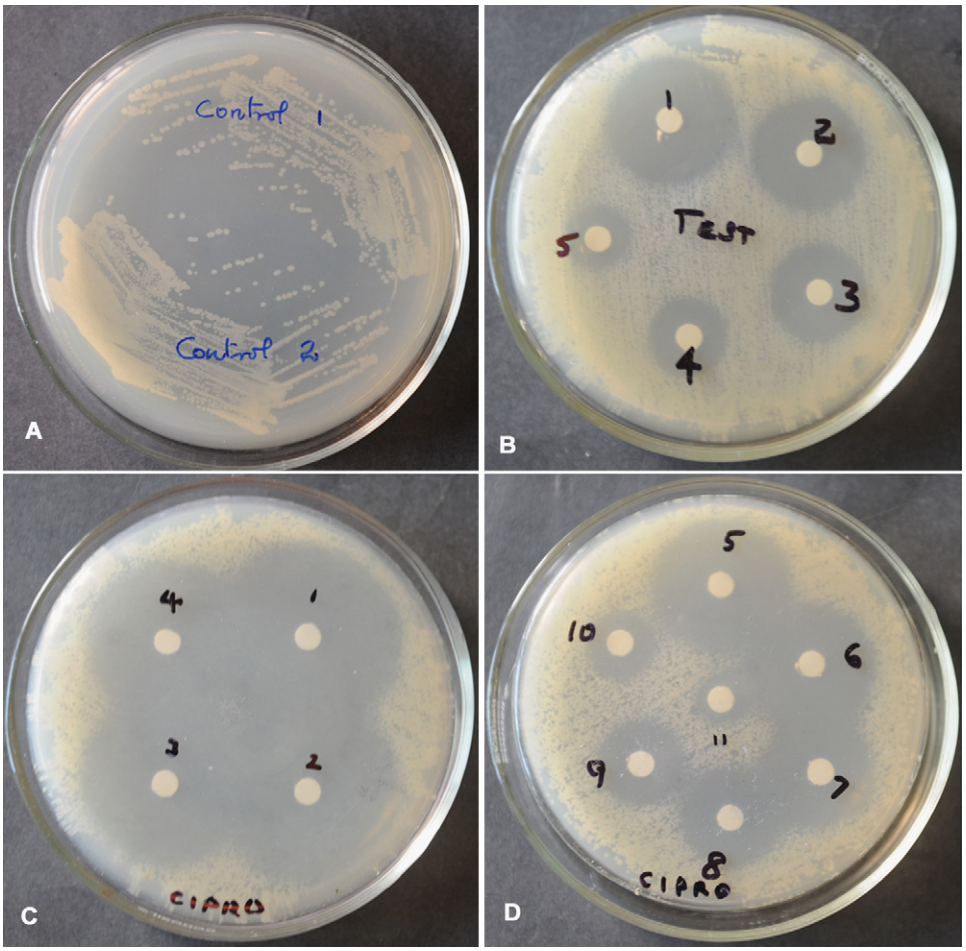


Fig. 6. Inhibition of ATCC25922 E coli by (A) the extracts of control films (chitosan poly(NIPAAm) blend with no drug), (B) the released ciprofloxacin from the thiolated chitosan poly(NIPAAm) blend film (Table 2 for dilutions), (C) different concentrations of Sigma Ciprofloxacin (Table 2 for concentrations).

als in contact with such cells would preserve the morphological aspects of the cells to larger extent, while the materials with cytotoxicity would cause the cells to undergo lysis or degeneration and lose the spindle shape and become more rounded. The blend film in contact with L929 fibroblast cells does not cause any cell lysis, degeneration or loss of the spindle shape morphology confirming that they are non-cytotoxic.

4.8.2. Test on extract method

Fig. 4C is the trypan blue stained image of the 1 week extract (100%) of the CH-T-PNi blend film. None of the cells have turned blue colour confirming that all the cells are viable on contact with the extract of the blend films. Hence it is proved that both the material and its degradation products if any are non-cytotoxic.

4.8.3. MTT assay

Quantitative assessment of the cytotoxicity by MTT assay of cells on contact with the different dilutions (100, 50 and 25%) of 1 week extracts of the blends showed 99–100% metabolically active cells compared to cells without the materials (negative controls) for 24 h of contact. The results showed are the average of three replicate experiments. Fig. S5 (supplementary data) shows the MTT reduction for 24 h contact of the microsphere extract obtained from 1 week incubation of the blend films in tissue culture medium at 37 °C along with the reduction observed for the positive and negative controls. Statistical analysis shows that compared to positive control the % viability of the different concentrations of the extract is statistically significant.

4.9. Ciprofloxacin uptake and its controlled release by the blend film

The uptake of ciprofloxacin by the CH-T-PNi film was assayed as detailed in Section 2. The 1 cm^2 size films were kept in 1 ml of a 10 mg/ml drug solution for a period of 24 h. The drug uptake was found to be 3.52 ± 0.2 mg/cm^2 for the blend films. The cumulative drug release percent of the film was estimated as 77.4 ± 3.8% of the incorporated drug when studied for a period of 48 h. An initial burst release of 35% was observed and after that the system followed a sustained release for 48 h as depicted in Fig. 5. When the drug release at different time intervals is compared with one another the values were found to be statistically significant.

4.10. Determination of antibacterial activity

Fig. 6A–D shows the microbial inhibition by the extracts prepared from the control film (the blend films without drug), 1:1 dilutions of the extracts of the drug loaded film and different concentrations of the Sigma grade ciprofloxacin, respectively. The details of dilutions of the test sample and concentrations of Sigma ciprofloxacin are given in Table 2 as serial numbers 1, 2, 3, etc. These numbers correspond to the numbers on the petri plates in Fig. 6B–D. Table 2 also shows the zone size of the 6 mm discs incorporated with different quantity of Sigma grade ciprofloxacin and the ciprofloxacin released from the test samples prepared in tube dilution method.

Table 2
The zone size of the 6 mm discs incorporated with different quantity of Sigma grade ciprofloxacin and the ciprofloxacin released from the test samples.

S. no.	Ciprofloxacin (μg/disc)	Zone size (mm)	Test sample (dilutions)	Zone size (mm)
1	0.125	40	1/2	25
2	0.06	38	1/4	23
3	0.03	35	1/8	20
4	0.015	32	1/16	15
5	0.0078	30	1/32	13
6	0.0039	28	1/64	8
7	0.0019	25	1/128	6.5
8	0.00097	23	1/256	–
9	0.00048	20	1/512	–
10	0.00024	18	1/1024	–

The MIC of ATCC 25922 E coli against Sigma grade ciprofloxacin was found to be <0.25 μg by Vitek 2 system and 0.02 μg/ml by tube dilution method. From the figure it is apparent that the extracts of the control films do not show any inhibition for the bacteria ATCC25922 E coli. A zone size of 25 mm is shown by 0.0019 μg/disc of Sigma grade ciprofloxacin while the test sample of 1/2 dilution showed the same zone size (Fig. 6B). The test samples showed inhibition of Ecoli up to a dilution factor of 1/32 which corresponds to an amount of <0.00024 μg/disc ciprofloxacin as per the zone size exhibited by Sigma grade ciprofloxacin.

5. Conclusions

A mucoadhesive, thermo responsive and drug releasing wound dressing is synthesized by polymerizing NIPAAm in the presence of thiolated chitosan. The film exhibited adequate mechanical property to be used as a wound/burn dressing and was found to be cytocompatible. The films were found to be antibacterial as they release the entrapped antibiotic in a controlled manner for a period of more than 48 h.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.08.042.

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