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Research Article

Synthesis and Evaluation of the Properties of Chitosan-Cellulose Whisker Microparticles for Controlled Release of Isoniazid

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Abstract

Cellulose whisker (CW) was synthesized from filter paper by acid hydrolysis. Chitosan –CW microparticles were prepared by microemulsion technique using isoniazid as the model drug and glutaraldehyde as the crosslinker. The microparticles were characterized by Fourier Transmission Infra-red Spectroscopy (FTIR), X-ray diffractometry (XRD), Scanning electron microscopy (SEM), Transmission emission microscopy (TEM). The effect of cellulose whisker and glutaraldehyde on the microparticles were assessed with regard to swelling, encapsulation efficiency and consequently on the release of isoniazid in different mediums. The drug release mechanism was studied for different time periods by UV-visible spectrophotometer. Cytotoxicity test was performed by MTT assay analysis. The results entailed that the microparticles can be exploited as potential drug carrier for controlled release applications.

Keywords: infrared spectroscopy; X-ray diffraction; transmission electron microscopy (TEM)
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Introduction

Drug delivery is attracting much attention as an interdisciplinary field of research in pharmaceutical industry. Controlled release methodologies are gaining more popularity in contemporary medicines. Various drug delivery systems have been formulated using different types of materials such as micelles, lipid vesicles, micro particles and hydrogels [1]. A drug delivery system may be a polymeric matrix incorporated with a drug. Polymeric drug delivery systems can deliver drugs at a constant rate over an extended period of time [2]. In the last few decades natural biodegradable polymers are largely used for drug delivery applications owing to their properties such as low toxicity, easy availability and cell biocompatibility [3, 4]. Among the natural polymers, polysaccharides have received increasing attention because of their outstanding physical and biological properties [5]. Chitosan plays an important role as drug delivery systems. Chitosan is a linear aminopolysaccharide composed of randomly distributed (1→4) linked D-glucosamine and N-acetyl-D-glucosamine units and is obtained by the deacetylation of chitin, a prevalent natural polysaccharide found in the exoskeleton of crustaceans such as crab and shrimp[6]. It can also be obtained from some microorganisms and yeasts. Chitosan is a mucoadhesive polycation polymer at acidic pH which is not noxious and biocompatible [6-9]. Chitosan has also fungicidal effect, wound healing properties and reduces cholesterol level [10]. Owing to its cationic nature chitosan has good mucoadhesive and membrane permeation enhancing properties [11].

Cellulose whiskers (CW) are anisometric nanofillers. They act as reinforcing elements. Cellulose whiskers consist of slender parallelepiped rods with nanometric lateral dimensions, high aspect ratios and large surface areas, and also have a renewable character. They have high tensile modulus [12]. CW have largely been employed as fillers in several kinds of polymeric matrixes from aqueous suspension, giving rise to very strong and tough interconnected networks of hydrogen bonded whiskers [13]. CW is prepared by dissolving the amorphous or less ordered regions of the microfibrils by acid degradation [14-15]. Major applications of CW in pharmaceutical field are based on its mechanical properties. CW is used in development of artificial ligaments and tendons [16] and implantable purpose such as heart valves and vascular grafts [17]. Evidently, much research is not done with CW in the field of controlled release drug delivery.

Isoniazid is an antituberculosis drug which can be used as a model drug.

Various methods are used to prepare chitosan microparticles, such as, microencapsulation, coacervation, emulsion-droplet coalescence, reverse micelles, ionic gelation and self assembly chemical modification. Extensive research has been done to synthesize chitosan microparticles by coacervation process [18-21]. The synthesized microparticles can be chemically crosslinked with glutaraldehyde and genipin or physically with sodium tripolyphosphate, sulphate, citrate, etc. Varieties of crosslinkers are reported to be used for controlling the release behavior of drugs [22-23]. In this paper, efforts have been made to prepare isoniazid loaded chitosan-CW microparticles by coacervation method followed by crosslinking chemically with glutaraldehyde (GA). This report aims at the study of the effect of CW and GA on various properties of microparticles. To our knowledge, there is no report on the use of CWs in microparticles for controlled drug delivery applications.

Materials and Methods

Materials

Medium molecular weight chitosan, Isoniazid, Histopaque 1077 and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) were obtained from Sigma Aldrich, Germany. Cellulose whisker used as one of the reinforcing agent were extracted from Whatman filter paper No. 1 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, U.K.). H_2SO_4 (purity > 97%), dimethyl sulfoxide (DMSO, purity > 99%) and Na_2SO_4 were purchased from Rankem, New Delhi, India. Tween 80 and calcium chloride ($CaCl_2$) were obtained from Merck, India. Edible grade refined sunflower oil was purchased from local market, Tezpur, India. Glutaraldehyde (GA) was procured from Loba Chemi Pvt. Ltd, Mumbai, India. RPMI 1640 and fatal bovine serum (FBS) were procured from HiMedia Laboratories (Mumbai, India). The rest of the chemicals were of analytical grade and used as such received.

Preparation of Cellulose Whisker (CW):

Cellulose whisker was synthesized from whatman filter paper by following the method of Zhang et al. with slight modification [24]. Whatman filter paper (No. 1) was sieved through a test sieve (Scientific Engineering Corporation, New Delhi, India) outfitted with 100 mesh screen to obtain very small size. The resultant cellulose powder (1g) was pretreated by taking into a beaker and was dispersed in 5 M NaOH solution. The mixture was stirred at 65 $^{\circ}$ C for 6 h. The slurry thus obtained was filtered and thoroughly washed with distilled water until neutral pH was obtained and dried in vacuum oven. 100 mL of DMSO was added to the dried cellulose. The slurry was then sonicated for 1 h, filtered, washed with 500 mL of distilled water and vacuum dried in oven.

Then, 1 g of the obtained cellulose was dispersed in 25 mL of 30% (v/v) H_2SO_4 in a three-necked (250 mL) round bottom flask equipped with a mechanical stirrer and a thermometer. The flask was then placed in a water bath, and its contents were stirred vigorously for 12 h while the temperature was maintained at 60 $^{\circ}$C. The suspension was then diluted with 75 mL of distilled water and sonicated for 1 h.

The dispersion was centrifuged at nearly 8000 rpm for 30 min and washed with distilled water until the pH of the supernatant liquid was nearly 7. The residues (cellulose whiskers) separated after high-speed centrifugation was collected and freeze-dried.

These cellulose whiskers (CW) were further used for the preparation of the microparticles.

Preparation of Chitosan –CW microparticles:

Microspheres of Chitosan composite were made by microencapsulation method as reported by Devi et al. with modifications [25]. In this method 1% (w/v) chitosan solution was prepared in water containing 0.5 % (v/v) acetic acid and measured amount of prepared cellulose whisker was dispersed in the chitosan solution along with 0.005 mL

Tween 80 (surfactant) and stirred for 1 h and then sonicated for 20 min. In another beaker, known amount of (250 mL) sunflower oil was taken. This oil was stirred for approximately 10 min. Under stirring condition, 50 ml of 1% chitosan solution containing dispersed cellulose whisker was added drop

wise to the beaker containing oil to form water in oil emulsion. After this, a known amount of Na_2SO_4 solution (20% w/v) was added slowly to the beaker to obtain the microparticles.

The optimum ratio of chitosan and Na_2SO_4 at which complete phase separation occurred was 1:2. The beaker containing the microspheres was left to rest for approximately 15mins under constant stirring condition. The system was then brought to 5-10 ℃ to harden the microsphere. Crosslinking of the microspheres was achieved by slow addition of certain amount of glutaraldehyde (GA) solution. The temperature of the beaker was then raised to 45 ℃ and stirring was continued for another 6 h to complete the crosslinking reaction. The beaker was then brought down to room temperature slowly while stirring. The microspheres were filtered through 300-mesh nylon cloth, washed with acetone rapidly to remove oil, if any, adhered to the surface of microspheres. This was further washed with distilled water until the pH of filtrate reached neutrality. The microspheres were then dried in open air. The dried microspheres were then dipped in isoniazid solution (0.5–10% w/v) for different time period (20–120 min), filtered through 300-mesh nylon cloth, and quickly washed with water to remove isoniazid, if any, adhered to the surface. The isoniazid-encapsulated microspheres were again freeze-dried and stored in a glass ampule in a refrigerator.

A series of seven samples were prepared for the present study as represented in Table 1.

Table 1 Recipes for the formation of different isoniazid loaded chitosan-CW microparticles crosslinked with isoniazid

Sample code	(Chitosan) % w/v (amount in g in 50 ml water)	CW (% w/w) w.r.t Chitosan (amount in g in 50 mL water)	GA (% v/w) w.r.t. CMC (amount in mL)	Isoniazid (g)	Tween 80 (mL)
C/CW0/GA50	1 (0.5)	0 (0.00)	0.25	0.1	0.005
C/CW1/GA50	1 (0.5)	1 (0.005)	0.25	0.1	0.005
C/CW3/GA50	1 (0.5)	3 (0.015)	0.25	0.1	0.005
C/CW5/GA50	1 (0.5)	5 (0.025)	0.25	0.1	0.005
C/CW5/GA10	1 (0.5)	1 (0.025)	0.05	0.1	0.005
C/CW5/GA30	1 (0.5)	1 (0.025)	0.15	0.1	0.005
C/CW5/GA70	1 (0.5)	1 (0.025)	0.35	0.1	0.005

Characterizations

Fourier Transmission Infra-red Spectroscopy (FTIR) study

FTIR spectra of microparticles were taken in Nicolet (model Impact-410) spectrophotometer. The microparticles were grounded to powder, mixed with KBr and spectra were recorded in the range of 4000 – 400cm^{-1}.

X-ray diffraction (XRD) study

The degree of intercalation of Chitosan-CW microparticles were examined by X-ray diffractometry. It was carried out in a Rigaku X-ray diffractometer (Miniflax, UK) using CuKα (λ=0.154 nm) radiation at a scanning rate of 1^o/min with an angle ranging from 2^o to 50^o of 2θ. The crystalline index of cellulose whisker, C_{Ir}, was calculated using the following equation [26]

$$C_{Ir}\ (\%) = [(I_{002} - I_{am})/ I_{am}] \times 100$$

where I_{002} is the intensity of lattice peak diffraction and I_{am} is the peak intensity of the amorphous fraction.

Particle size determination

Particle size was determined by dynamic light scattering (DLS) analyzer (model DLS—Nano ZS, Zetasizer, Nanoseries, Malvern Instruments).

Scanning electron microscopy (SEM) study

The samples were mounted on a brass holder, sputtered with platinum. The surface morphologies of Chitosan-CW microparticles loaded with isoniazid were studied by using Scanning Electron Microscope (JEOL JSM – 6390LV) at an accelerated voltage of 5-10 kV.

Transmission emission microscopy (TEM) study

The dispersion of the CW in chitosan microparticles was examined by using Transmission Electron Microscope (JEOL JEM-2100) at an accelerated voltage of 100 kV

Calibration curve of isoniazid

A calibration curve is essential to estimate the release rate of drug from microparticles in the suitable solvent medium. Calibration curve was drawn as per the procedure [25].

A known concentration of isoniazid (in double distilled water) was scanned in the range 200 – 400 nm by using UV− Visible spectrophotometer (UV-2001Hitachi, Tokyo, Japan). A sharp peak at 262 nm was observed for isoniazid having concentration in the range of 0.001 – 0.01g/100 mL. The absorbance values at 262 nm obtained with respective concentration were recorded and plotted. From this calibration curve, the unknown concentration of isoniazid was obtained by knowing the absorbance value.

Calculation of Percentage Encapsulation efficiency and percentage drug loading of the microparticles

A known amount of accurately weighed microspheres was grounded in a mortar, transferred to a volumetric flask containing 100 mL of water, and kept overnight with continuous stirring to dissolve the drug inside the microspheres. The solution was collected and the drug inside the microspheres was determined using UV-Visible spectrophotometer. The encapsulation efficiency and drug loading (%) was calculated by using the calibration curve and the following formula [25].

$$Encapsulation\ efficiency,\ (EE)\ (\%) = (w_1/w_2) \times 100$$

w_1 =actual amount of drug encapsulated in a known amount of microparticles

w_2 = amount of drug introduced in the same amount of microparticles

Drug loading (%) = $(w_3-w_4)/w_4$ x 100

Here, w_3 = weight of swollen microsphere in drug solution

w_4 = initial weight of dry microsphere

Water Uptake Studies

Water uptake studies were performed in both phosphate buffer (pH 7.4) and 0.1N HCl solution (pH 1.2) according to the procedure described in the literature [25].

Microparticles (0.1 g) were taken in a pouch made of nylon cloth. The empty pouch was first conditioned by immersing it in either 0.1 N HCl (pH 1.2) or phosphate buffer (pH 7.4) for different time periods (1-8 h). The pouch containing the microparticles was immersed in a similar way in either 0.1 N HCl (pH 1.2) or phosphate buffer (pH 7.4) for the similar time periods. The weights of wet microparticles after a definite time period were determined by deducting the respective conditioned weight of the empty nylon pouch from this. Water uptake (%) was determined by measuring the change in the weight of the microparticles. The percentage of water uptake for each sample determined at time‘t’ was calculated using the following equation.

Water uptake (%) = $[(w_2 - w_1) / w_1] \times 100$

where, w_1 is initial weight of microparticles before swelling,

and w_2 is the final weight of microparticles after swelling for a predetermined time ‘t’.

The experiments were performed in triplicate and represented as a mean value.

In vitro drug release studies

To study the release profile of the isoniazid loaded chitosan-CW microparticles, dried drug loaded samples were immersed in a solution of different pH namely 1.2 and 7.4 and stirred continuously. At scheduled time interval, 5mL solution was withdrawn, filtered and assayed spectrophotometrically at 262 nm by using UV-Visible spectrophotometer for the determination of cumulative amount of drug release upto a time *t*. To maintain a constant volume, 5 mL of the solution having same pH was returned to the container [27-28]. Each determination was carried out in triplicate.

Isolation of Lymphocytes, culture and treatment

Chicken blood was collected from local butcher shop. It was diluted in the ratio of 1:1 with phosphate buffer saline (PBS) and encrusted 6 mL into 6 mL histopaque (1.077 g/mL). The isolation of lymphocytes and the study of cell viability were conducted as per the procedure cited in the literature [29]. Lymphocytes were isolated from the sample after centrifugation for 30 min at 400 g, washed with PBS and finally with serum free media individually through centrifugation for 10 min at 250 g. Cell pellets were then suspended in PBS and cell viability was noticed by Trypan blue exclusion method using haemocytometer. Cell viability more than 90 % was used for subsequent study.

Aliquots of 200 µL of isolated cells were cultured plate in RPMI supplemented with 10% heat inactivated fatal bovine serum (FBS). Initially cells were maintained for 4 h in RPMI without FBS at 37°C in 5 % CO_2 incubator. Cells were then treated with as per experimental requirements and maintained in presence of FBS for 6 h, 12h and 24 h.

Cytotoxicity experiments

Cytotoxicity assay was executed by measuring the viability of cells according to the method as described by Denizot and Lang [30]. The prime component (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) (MTT) is yellowish in color and mitochondrial dehydrogenase of viable cells slice the tetrazolium ring, yielding purple insoluble formazan crystals which were dissolved in suitable solvent. In this report, DMSO is used as the control solvent. The ensuing purple solution was spectrophotometrically calculated. Changes in cell number resulted in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material. Briefly, after treatments, cells were treated with 10 % of MTT for 2 h followed by dissolving the formazan crystals in solvent and measuring the absorbance of solution at 570 nm. The absorbance of control cells at 6, 12 and 24 h were separately set as 100% viability and the values of treated cells were calculated as percentage of control.

Statistical analysis

All the data were expressed as means ± SD. Results were statistically analyzed by student's t test for significance difference between group mean using GraphPad software [31]. The significant difference between the experimental and the control group was set at different levels as $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results and Discussion

Fourier Transmission Infra-red Spectroscopy (FTIR) study:

FTIR spectra of Cellulose (ground filter paper), chitosan, CW, isoniazid and isoniazid loaded chitosan-CW microparticles are shown in Fig. 1.

The successful synthesis of CW from Whatman filter paper was confirmed by FTIR analysis. The FTIR spectra of ground Whatman filter paper (curve a) shows a broad peak at 3426 cm^{-1} which is attributed to OH stretching. The peak at 2905 cm^{-1} was due to symmetric CH stretching. A strong band at 1639 cm^{-1} was observed which is attributed to stretching vibration resulting from H-O-H intermolecular linkages. Bands at 1447 cm^{-1} and 1333 cm^{-1} were due to symmetric CH_2 bending and wagging respectively. Peaks at 1379 and 1259 cm^{-1} were attributed to C-H bending vibrations. Band at 1179 cm^{-1} and 896 cm^{-1} were assigned for C-O-C stretching frequency at the β-(1→4) glycosidic linkages. A small peak at 1119 cm^{-1} is observed due to the in plane ring of the β-(1→4) glycosidic linkages. Strong peak at 1049 cm^{-1} was attributed to the C-O stretching at C-3.

The peaks assigned at 789 cm^{-1} and 712 cm^{-1} were the characteristic absorption of cellulose I_α and I_β, respectively [32].

The FTIR spectra of CW (curve b) showed that the peaks were almost similar to that of the untreated ground filter paper. The changes are observed only in the intensities of the peaks. The intensities of the same peaks appeared at 1379 cm^{-1}, 1119 cm^{-1} and 896 cm^{-1} increased whereas the intensities of the peaks found at 2905 cm^{-1}, 1259 cm^{-1}, 1639 cm^{-1} and 1333 cm^{-1} did not alter much [32].

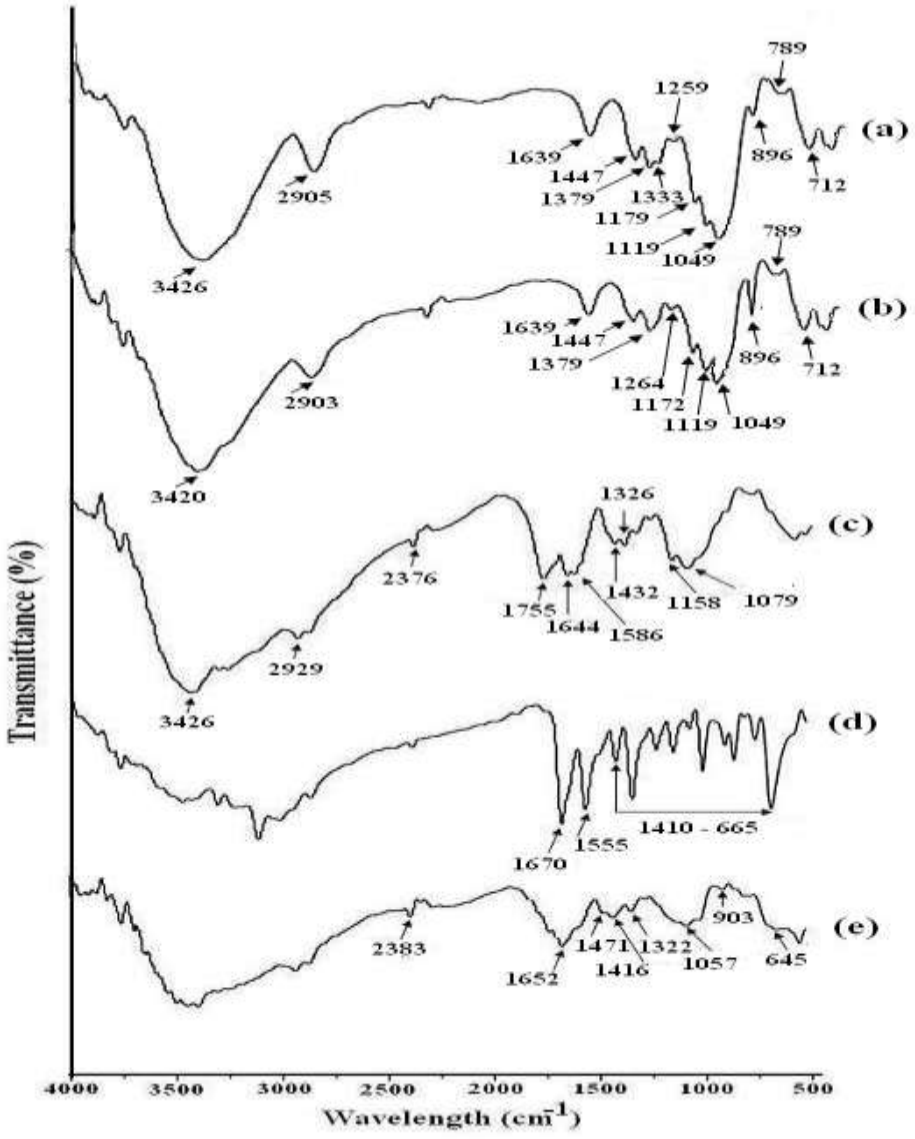

Figure 1 FTIR spectra of (a) Cellulose, (b) CW, (c) Chitosan, (d) Isoniazid, and (e) C/CW5/GA50

The basic absorption bands of chitosan (curve c) appeared at 3442 cm^{-1} (OH stretching and NH stretching, overlapped), 2939 cm^{-1} (CH stretching). The absorption peaks of amide I and amide II appeared at 1639 cm^{-1} (C=O stretching) and 1455 cm^{-1} (N-H in plane deformation coupled with C≡N stretching) respectively. The other notable peaks were found to appear at 1156 cm^{-1} (bridge –O- stretching), and 1062 cm^{-1} (-CO stretching) [33].

Curve d represents the spectrum of isoniazid. The absorption peaks appeared at 1664 and 1551 cm^{-1}were due to the amide I (C=O stretching) and amide II (N-H bending of secondary amide group) respectively. Besides this, multiple peaks appeared in the range 1410-669 cm^{-1} [34].

The appearance of all the characteristic peaks of chitosan, CW and isoniazid in the spectrum of isoniazid loaded chitosan-CW microparticles (curve e) suggested the incorporation of isoniazid and CW into the microparticles.

X-ray diffraction (XRD) study

The XRD diffractograms of the ground filter paper, CW, Chitosan, isoniazid and isoniazid loaded chitosan-CW microparticles are shown in Fig. 2.

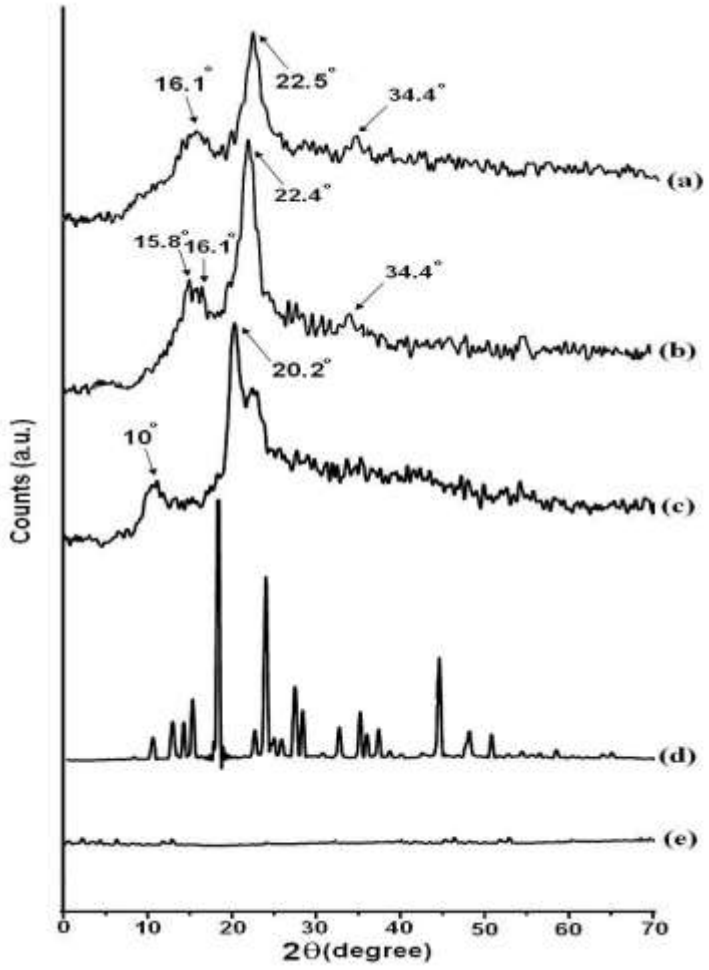

Figure 2 XRD patterns of (a) Cellulose, (b) CW, (c) chitosan, (d) Isoniazid, and (e) C/CW5/GA50

For CW, diffraction peaks were obtained at 2θ angles of 15.8°, 16.1°, 22.4°, and 34.4°corresponding to the (1−10), (110), (002), and (004) crystallographic planes, respectively. The intensity of peak for the (002) plane (2θ = 22.4°) of CW was much sharper than that of the untreated ground filter paper. The intensity of the peak appearing at 2θ = 16.1° (110 plane) for CWs is sharper than that of the peak of ordinary filter paper [32]. From the diffractograms it was observed that the crystallinity of CW increased, as the peak intensity of the CWs was greater than that of the untreated ground filter paper.

The crystallinity index of CW was calculated from the formula

$$C_{Ir}\ (\%) = [(I_{002} - I_{am})/ I_{am}]\ \times 100$$

where I_{002} is the intensity of lattice peak diffraction at 22.4° and I_{am} is the peak intensity of the amorphous fraction at 16.1°. The crystallinity index was found to be 72.8%

Chitosan showed two distinct crystalline peaks at 2θ= 10° and 20.2° due to (*020*) and (*100*) plane, respectively [Fig. 2c] [33].

Isoniazid (curve 2d) shows multiple peaks at 2θ=12° to 50° due to its crystalline nature. Similar type of diffractogram was reported by Maji et al.[35].

The characteristic peaks for CW and isoniazid were found to disappear in the diffractogram of chitosan-CW microparticles (curve 2e). These findings indicated that CW lost its structure in the microparticles either due to vigorous stirring or due to the presence of chitosan matrix. The findings also suggested the occurrence of a molecular level dispersion of isoniazid in isoniazid loaded chitosan-CW microparticles.

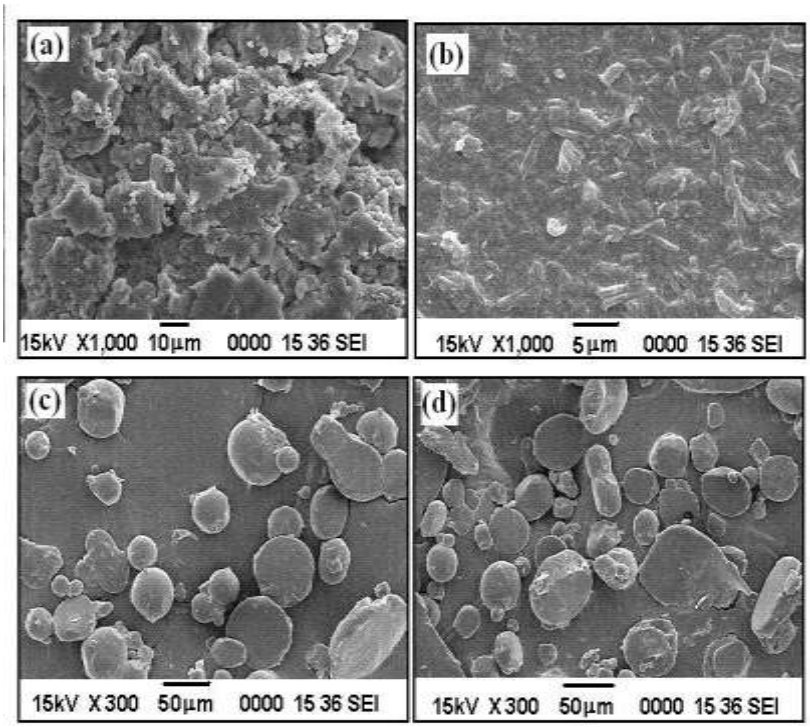

Figure 3 SEM micrographs of (a) Untreated filter paper, (b) CW, (c) C/CW0/GA50 and (d) C/CW5/GA50

Scanning electron microscopy (SEM) study

SEM analysis was performed to study the surface morphology of CW, chitosan microparticles and chitosan-CW microparticles loaded with isoniazid. Fig. 3(a-d) represents the SEM micrographs of filter paper, CW, isoniazid loaded chitosan microparticles and isoniazid loaded chitosan-CW microparticles respectively. The surface of the untreated filter paper is agglomerated [Fig. 3(a)]. The surface of CW (Fig.3b) was found to appear as small rod shaped and aggregated in nature [36]. Both isoniazid loaded chitosan microparticles which are devoid of CW [Fig. 3 (c)] and which are incorporated with CW [Fig.

3(d)] showed spherical shapes. But the surface of isoniazid loaded chitosan-CW microparticles was more rough compared to isoniazid loaded chitosan microparticles suggesting good adhesion between CW and chitosan matrix.

Transmission electron microscopy (TEM) study

TEM micrographs of isoniazid loaded chitosan microparticles devoid of CW and with CW are shown in Fig. 4a and 4b respectively. Fig. 4b showed the presence of network structure of CW in the chitosan matrix and the CW chains are bundled together. This could be ascribed to the formation of hydrogen bonding between the CW and the chitosan matrix. These types of structure were not observed in Fig. 4a. The results indicated that CW were incorporated and dispersed in the chitosan matrix. Similar results were obtained by Maji et al.[32]

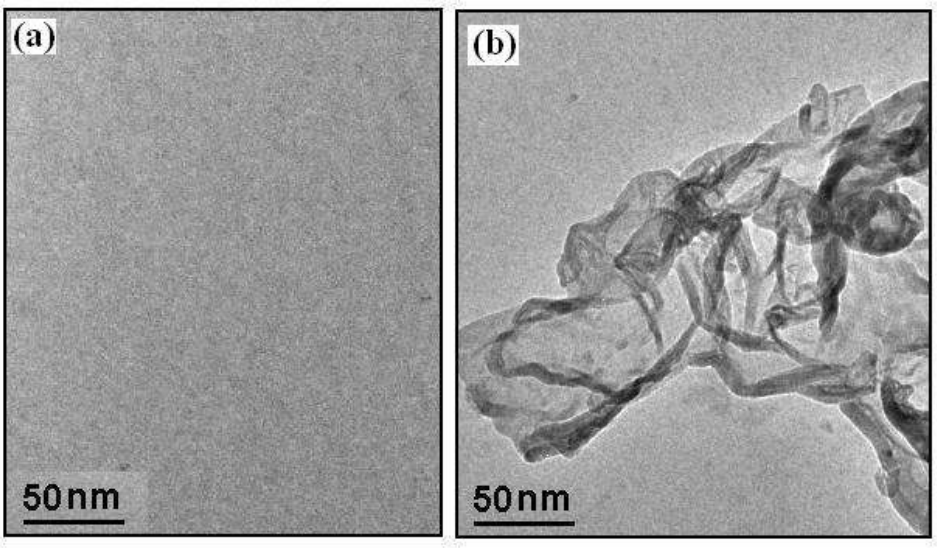

Figure 4 TEM micrographs of chitosan microparticles (a) without CW range and (b) with CW at 50 nm scale respectively

Effect of variation of CW and GA concentration on the different properties of isoniazid loaded chitosan CW microparticles

The results showing the effect of variation of CW and GA concentration on different properties of chitosan microparticles are shown in Table 2. The encapsulation efficiency and drug loading of CW free crosslinked microparticles was found to be higher compared to that of CW containing crosslinked microparticles. The encapsulation efficiency decreased with the increase in CW content in the microparticles. This could be attributed to the presence of the network structure of CW which restricts the diffusion of drug in the microparticles. The –OH groups of CW could interact with the $–NH_2$ group of chitosan and –CHO groups of GA resulting in extension of the polymer chains. The network structure of CW also hindered the movement of the intercalated polymer chains freely. The interference offered by the network structure of CW layers was absent in CW free crosslinked microparticles. Hence, it showed higher encapsulation efficiency.

Similarly, at a fixed CW content, the encapsulation efficiency of microparticles was found to further decrease with the increase in the glutaraldehyde (GA) concentration. With the increase in GA content, the crosslinking increased and the surface of the microparticles get hardened. As a result, less amount of drug can diffuse through the microparticles and consequently the encapsulation efficiency and drug loading decreased.

The average diameter of the microparticles was obtained in the range 823-919 nm. The variation in CW concentration did not significantly affect the particle size. However, the average diameter showed a decreasing trend on increasing the GA concentration. The amino groups present in chitosan interacted with the hydroxyl group of CW and GA. With the increase in the concentration of GA, the availability of free amino groups on microparticles reduces due to which the microparticles became more compact and hence the diameter would be less.

Zeta potential values of the microparticles were found in the range 43 to 55 mV indicating good stability of the microparticles. The surface of the microparticles was positively charged due to the presence of residual amino groups. With the incorporation of CW in chitosan matrix the surface charge decreases. The reduction in surface charge might be due to the increased electrostatic interaction between the protonated amino groups of chitosan and hydroxyl groups of CW.

Table 2 Effect of variation of CW and GA concentration on the different properties of chitosan microparticles

Sample code	Encapsulation efficiency (%)	Drug loading efficiency (%)	Average diameter (nm)	Zeta potential (mV)	Zeta potential of the same formulations without drug (mV)	Zeta potential of nanoparticles after 3 hours of drug release (mV)
C/CW0/GA50	72.10(±0.02)	29.39(±0.08)	889.4 (± 15)	55.32 (±0.03)	35.05 (±0.01)	48.23(±0.04)
C/CW1/GA50	69.83(±0.04)	26.61(±0.04)	889.7(±12)	53.49(± 0.1)	34.12(±0.03)	48.07(±0.08)
C/CW3/GA50	67.43(±0.04)	24.18(±0.01)	886.1 (± 19)	53.21 (±0.1)	34.08(±0.07)	46.23(±0.01)
C/CW5/GA50	67.10(±0.01)	22.27(±0.01)	856.3 (± 13)	51.92(± 0.03)	36.22(±0.06)	47.34(±0.03)
C/CW5/GA10	70.21(±0.03)	25.63(±0.02)	919.2 (± 15)	43.87(± 0.03)	32.11(±0.06)	41.23(±0.04)
C/CW5/GA30	70.09(±0.02)	22.58(±0.01)	902.9(±16)	50.63 (±0.06)	33.90(±0.01)	45.12 (±0.04)
C/CW5/GA70	65.13(±0.02	19.19(±0.01)	823.7(±10)	52.13 (±0.01)	36.41(±0.03)	48..34(±0.01)

*each value represents average of five readings, standard deviation in parenthesis

However, the zeta potential values increased as the GA concentration increased from 10 to 70%. The increase in the zeta potential indicated that the stability of the microparticles increased with the increase in the concentration of GA and they would not aggregate in acidic or basic medium [34]. All the zeta

potential values were in the stable zone indicating that the synthesized microparticles are highly stable. The zeta potential values of the microparticles without drug showed lower value compared to the zeta potential of the microparticles after loading of drug. This showed that the stability and surface charge of the microparticles enhances on the incorporation of the drug. The increase in the zeta potential with the incorporation of the drug suggested that some of the drug polymer association was surface-adsorbed. Thus, a part of the drug was encapsulated within the chitosan matrix and the rest was adsorbed on the surface of the microparticles.

Swelling Study:

The effect of pH on the percentage swelling of isoniazid loaded microparticles at two different pH namely, 1.2 and 7.4 are shown in Fig. 5. It was observed that the swelling of isoniazid loaded chitosan-CW microparticles was more in gastric pH (1.2) than in intestinal pH (7.4). At lower pH, the free amine groups become protonated and generated a repulsive force between the adjacent positively charged polymer chains causing the swelling of the polymer and consequently diffusion of more amount of drug out of the polymer matrix [37]. In alkaline pH, the inbuilt hydrophobicity of chitosan nanoparticles prevented them from faster swelling [38].

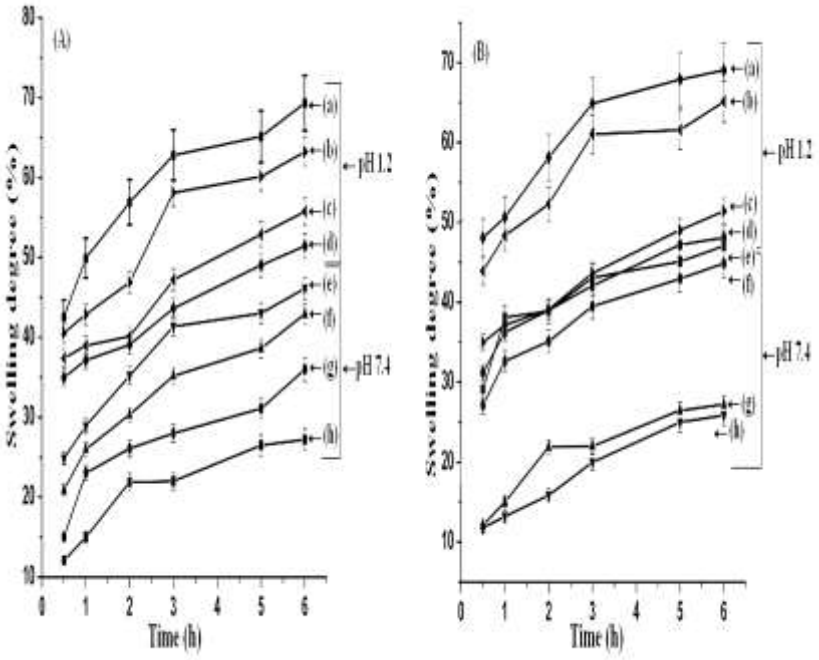

Figure 5 Percentage swelling degree at pH 1.2 and 7.4 ,(A){ (a)C/CW0/GA50, (b) C /CW1/GA50, (c) C/CW3/GA50, (d) C/CW5/GA50, (e) C/CW0/GA50, (f) C /CW1/GA50, (g) C/CW3/GA50, (h) C/CW5/GA50} and (B) {(a) C/CWM5/GA10, (b) C/CW5/GA30, (c) C /CW5/GA50, (d) C /CW5/GA70, (e) C/CWM5/GA10, (f) C/CW5/GA30, (g) C /CW5/GA50, (h) C /CW5/GA70}

Fig. 5 (A) showed that with the increase in the concentration of CW, the percentage swelling degree decreased. Water absorption decreased by the presence of dispersed phase of CW into the chitosan matrix of the microparticles. CW particles acted as a blockade for water molecules and decreased the water diffusion through the crosslinked chitosan-CW microparticles. Similarly, microparticles containing higher concentration of GA (Fig. 5B) swelled less due to higher crosslinking densities and less availability of the polar groups [32].

Furthermore, the percentage swelling degree was found to increase with the increase in time. With the increase in the time period, higher amount of the solvents can penetrate into the chitosan matrix, resulting in the increase in the percentage swelling degree.

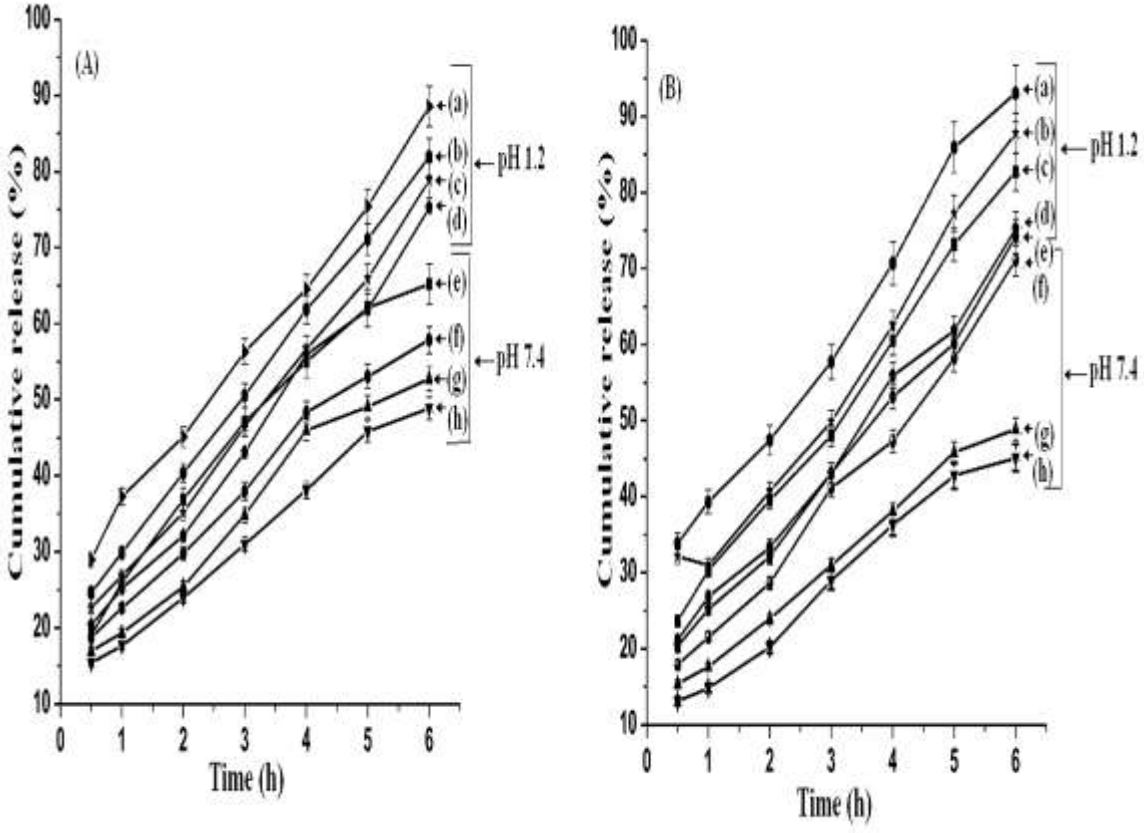

Figure 6 Cumulative release (%) at pH 1.2 and 7.4 ,(A){ (a)C/CW0/GA50, (b) C /CW1/GA50, (c) C/CW3/GA50, (d) C/CW5/GA50, (e) C/CW0/GA50, (f) C /CW1/GA50, (g) C/CW3/GA50, (h) C/CW5/GA50} and (B) {(a) C/CWM5/GA10, (b) C/CW5/GA30, (c) C /CW5/GA50, (d) C /CW5/GA70, (e) C/CWM5/GA10, (f) C/CW5/GA30, (g) C /CW5/GA50, (h) C /CW5/GA70}

In vitro Release Studies:

The drug release profile of the microparticles at two different pH namely 1.2 and 7.4 are shown in Fig. 6. The cumulative release (%) of isoniazid from chitosan-CW microparticles was pH dependent. The cumulative release (%) of isoniazid decreased with the increase in the pH of the medium. The major factors governing the release of isoniazid from microparticles were the swelling nature of the polymer and solubility of the drug in the solvent medium. The difference in release profile was due to the difference in the swelling behavior of chitosan in gastric and intestinal pH [39]. Chitosan swelled more in gastric pH compared to intestinal pH medium. The faster drug release rate in lower pH medium was due to the unstable microparticles structure, caused by the protonation of amino groups of chitosan in lower pH [37]. The solubility of isoniazid increased at acidic pH due to its basic nature as reported in literature [40]. Acidic pH of the medium favored both the swelling of the polymer and solubility of the drug.

It was also observed that the cumulative release (%) of isoniazid decreased with the increase in CW content (Fig. 6 A) and increased with the increase in the period of time. The percentage swelling of the microparticles decreased with the increase in the concentration of CW as stated earlier. Therefore, in order to aid the release of isoniazid the solvent particles could not diffuse properly to interact with the isoniazid molecules encapsulated in the microparticles. With the increase in time, the percentage degree of swelling increased and more and more solvent molecules could reach the drug molecule facilitated the release the isoniazid from the microparticles.

It was also seen that the cumulative release (%) of isoniazid decreased with the increase in the concentration of GA (Fig. 6 B). This was due to the increase in crosslinking density of the microparticles. With higher crosslinking, the surfaces of the microparticles get hardened. The solvent molecules cannot easily penetrate the surface of the microparticles to reach the drug and thus decreases in cumulative release (%) were observed.

Cytotoxicity Test

The effect of varying CW concentration (0-5%) and time (6, 12, and 24 h) on cell viability is shown in Fig 7. Fig. 7(a) showed that chitosan was not cytotoxic. Chitosan is used in pharmaceutical field due to its non toxic nature. It had high cell viability of around 98%. The synthesized CW was also found to be cell compatible and cell viability of CW was found around 92%. CW [Fig. 7(b)] was not at all cytotoxic to the cells as it was derived from cellulose. Similar types of observations were reported by Clift et al. [41]. The in vitro cytotoxicity study of Isoniazid [Fig. 7(c)] showed that Isoniazid was highly cytotoxic to the cells with a cell viability of only around 40%. This cell viability decreased with the increase of time (6 - 24h). It was observed that the cytotoxicity isoniazid loaded chitosan-CW microparticles decreased and cell viability increased. The cell viability increased with the increase in the CW [Fig. 7(d-f)] in the microparticles. Owing to the network structure of CW, the drug could not easily diffuse out to interact with the cell and hence an increase in cell viability was observed. As per expectation, the cell viability decreased with the increase in the time due to diffusion of more and more drug from the microparticles to the cell environment.

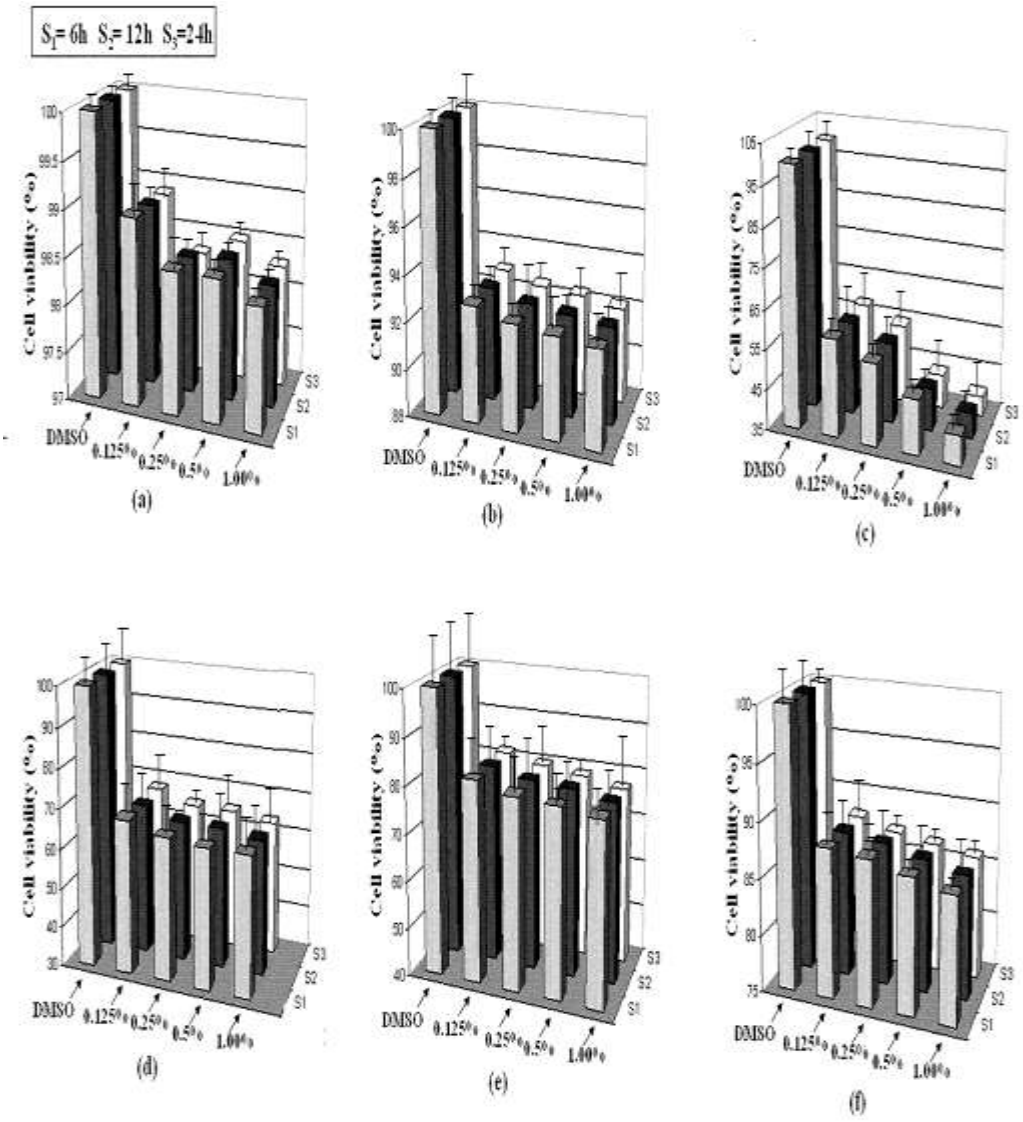

Figure 7 Cell viability study with variation of (a) Chitosan, (b) CW, (c) isoniazid, and (d) C/CW0/GA50 (e) C/CW1/GA50 (f) C/CW5/GA50 at 6 h, 12 h, and 24 h

Conclusions

This work demonstrated the successful preparation of CW and isoniazid loaded chitosan-CW microparticles by microencapsulation method. FTIR study indicated the successful preparation of CW and successful incorporation of CW and isoniazid in the microparticles. XRD results illustrated the increase in the crystallinity of CW compared to untreated ground filter paper. XRD results also showed

the molecular level dispersion of isoniazid in the chitosan-CW microparticles. XRD and TEM showed successful incorporation of CW in chitosan microparticles. SEM study demonstrated that both isoniazid loaded CW incorporated chitosan microparticles and only isoniazid loaded chitosan microparticles had spherical shape. The surface of isoniazid loaded chitosan-CW microparticles was more rough compared to isoniazid loaded chitosan microparticles suggesting good adhesion between CW and chitosan matrix Both the swelling and release of isoniazid from the microparticles were found to enhance with the decrease in the MMT and GA content. The percentage swelling degree and cumulative release increased in gastric pH compared to intestinal pH. Cytotoxicity study revealed that the synthesized CW was not cytotoxic and the microparticles containing CW was less cytotoxic than those of CW free nanoparticles.

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