

Development and Characterization of Biodegradable Chitosan Films for Local Delivery of Paclitaxel

Submitted: January 23, 2004; Accepted: June 22, 2004; Published: October 11, 2004.

Anand Babu Dhanikula,¹ and Ramesh Panchagnula¹

¹Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar 160062, India

ABSTRACT

Intratumoral and local drug delivery strategies have gained momentum recently as a promising modality in cancer therapy. In order to deliver paclitaxel at the tumor site in therapeutically relevant concentrations, chitosan films were fabricated. Paclitaxel could be loaded at 31% wt/wt in films, which were translucent and flexible. Physicochemical characterization of paclitaxel via thermal, spectroscopic, x-ray diffraction, and electron microscopy techniques revealed information on solid-state properties of paclitaxel as well as chitosan in films. While chitosan was in amorphous form, paclitaxel seemed to be present in both amorphous and crystalline forms in film. The polymeric dispersion of paclitaxel in poloxamer formed fibrous structures generating discontinuities in the film matrix, thereby leading to the introduction of perturbations in the packing arrangement of polymer chains. These films released only 10% to 15% of loaded paclitaxel by a burst effect under in vitro testing conditions, with lysozyme having no effect on the release. However, films softened after implantation in mice and lost integrity over time. The implantable delivery system is not only biodegradable but also well tolerated in vivo and hence, biocompatible as revealed by histological studies. The lack of formulation-induced local inflammatory responses of paclitaxel chitosan films suggests a new paradigm for localized chemotherapy based on implantable systems.

KEYWORDS: paclitaxel, local delivery, film, solid-state, histology, mice.

INTRODUCTION

In recent years, biodegradable polymeric systems have gained importance for design of surgical devices, artificial organs, drug delivery systems with different routes of administration, carriers of immobilized enzymes and cells, biosensors, ocular

Corresponding Author: Ramesh Panchagnula, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, Phase X, SAS Nagar, 160062 (Punjab) India. Tel: 91-172-2214682, 2214687. Fax: 91-172-2214692. Email: panchagnula@yahoo.com.

inserts, and materials for orthopedic applications.¹ These polymers are classified as either synthetic (polyesters, polyamides, polyanhydrides) and natural (polyamino acids, polysaccharides).² Polysaccharide-based polymers represent a major class of biomaterials, which includes agarose, alginate, carageenan, dextran, and chitosan.

Chitosan, $\beta(1,4)$ 2-amino-2-D-glucose, is a cationic biopolymer produced by alkaline N-deacetylation of chitin, which is the main component of the shells of crab, shrimp, and krill. Chitosan has found many biomedical applications, including tissue engineering, owing to its biocompatibility, low toxicity, and degradation in the body by enzymes such as chitosanase and lysozyme,³ which has opened up avenues for modulating drug release in vivo in the treatment of various diseases. These chitosan-based delivery systems range from microparticles to nanoparticles⁴ to gels⁵ and films.⁶ Further, gels and films of chitosan have been used for oral delivery of chlorhexidine digluconate in the treatment of fungal infections.⁷ In addition, chitosan has been extensively evaluated as a carrier of various antineoplastic agents such as 5-fluorouracil,⁸ mitoxantrone,⁹ cytarabine,¹⁰ and paclitaxel.¹¹

The film-forming property of chitosan has found many applications in tissue engineering and drug delivery by virtue of its mechanical strength and rather slow biodegradation.¹² Some drug-loaded chitosan films are emerging as novel drug delivery systems,¹³⁻¹⁴ and films appear to have potential for local sustained delivery of cancer chemotherapeutic agents. Following surgical removal of tumor, these implantable systems may be placed in the resection cavity to elicit a local response at the biophase; further, they may be secured by suturing at the site to prevent any displacement problems.

Though paclitaxel is the most extensively investigated anticancer drug in the last 3 decades, it is not a good option for the treatment of brain tumors after systemic administration.¹⁵ Successful treatment of malignant brain tumors is alarmingly negligible because antineoplastic agents, including paclitaxel, have limited access to the tumor site across the blood brain barrier when administered systemically. An alternative approach to systemic delivery of antineoplastic drugs is localized delivery from a polymer matrix. In the field of local delivery, carmustine-loaded Gliadel wafer (Guilford Pharmaceuticals, Baltimore, MD) fabricated from poly(carboxyphenoxy propane:sebacic acid) proved very promising in

clinical trials for the treatment of malignant glioma, increasing both survival and safety.¹⁶ The objective of this study was to develop a chitosan film-based local delivery system for sustained release of paclitaxel to tumor site after implantation. These films have been evaluated for the release of impregnated paclitaxel, characterized by physical techniques and microscopy, and examined for inflammatory reactions by histological examination after implantation in mice.

MATERIALS AND METHODS

Materials

Paclitaxel was a gift sample from Dabur India Ltd (Uttar Pradesh, India). Radioactive paclitaxel (¹⁴C) of specific activity 42.5 mCi/mmol, chitosan (≥85% deacetylated), lysozyme, and Tween 80 were purchased from Sigma (St Louis, MO). Poloxamer 407 was obtained as gratis sample from BASF (Ludwigshafen, Germany). Soya phosphatidylcholine and phosphatidylglycerol were kindly provided by Nattermann & Cie GmbH (Cologne, Germany). Absolute ethanol (ETOH) was procured from Merck KGaA (Darmstadt, Germany). Glycerol and glacial acetic acid were obtained from LOBA Chemie (Mumbai, India). High pressure liquid chromatography (HPLC)-grade methanol was obtained from (J.T. Baker, Madero, Mexico). Thiopentone sodium and gentamicin were of parenteral grade. All other reagents were analytical or reagent grade. Water obtained from ELGA purification unit (Marlow, UK) was used throughout the study. All animal experimentation was performed in accordance with protocols approved by the institutional animal ethical committee.

Preparation of Paclitaxel Chitosan Films

Because paclitaxel is a hydrophobic drug, 2 different approaches were used for its incorporation into chitosan films: one involved only phospholipids and the other used poloxamer 407 in presence of ETOH. Initially, a 10 mg/mL chitosan solution was prepared in 1% (vol/vol) acetic acid, and glycerol was included as a plasticizer at a chitosan:glycerol weight ratio of 2:1. In the first method, liposomes containing paclitaxel (spiked with radioactive component) were prepared by film hydration method using phosphatidylcholine and phosphatidyl glycerol (9:1, soya origin) at 6 to 12 mol% drug loading and were subsequently dispersed in chitosan solution. Then, film was cast by pouring the mixture on a glass plate (area 45.5 cm²) followed by drying under vacuum for 48 hours at 37°C. In the second method, required quantities of paclitaxel and poloxamer 407 were dissolved separately in 1 mL of ETOH and mixed together, and the ethanolic solution was added to chitosan solution and agitated to disperse paclitaxel. Subsequently, the homogeneous suspension was cast into film by the method described above

and dried at 60°C for 12 hours. After preparation, all films were stored in airtight containers for further studies.

Stability of Paclitaxel During Film Preparation

The following procedure was used to assess the stability of paclitaxel during the film preparation process. The prepared films were extracted twice with a solvent mixture of 1:1 acetonitrile and ETOH (vol/vol); the extract was evaporated; the residue obtained was reconstituted in mobile phase; and an aliquot was injected onto HPLC column. Stability-indicating chromatographic method was adopted for this purpose (Waters Corp, Milford, MA).¹⁷ The method consisted of a Symmetry C18 column (250 × 4.6 mm; 5 μm) run using a mobile phase of composition methanol:water (70:30 vol/vol) at a flow rate of 0.5 mL/min, a Waters pump (600 E), and eluants monitored with Waters photodiode array detector (996 PDA) at 227 nm.

Content Uniformity of Films

To ensure uniform distribution of paclitaxel in film, a content uniformity test was performed. Samples representing different regions within film were cut and weighed, and paclitaxel was extracted with a 1:1 solvent mixture of acetonitrile and ETOH (vol/vol) twice for 12 hours each time at room temperature. These extracts were pooled for liquid scintillation counting (EG&G Wallac, Turku, Finland).

Release Studies

A definite weight range of 10-15mg of film was cut and placed in a 1.5-mL capacity microcentrifuge tube containing 1 mL of release medium of the following composition at 37°C: phosphate buffered saline (140 mM, pH 7.4) with 0.1% sodium azide and 0.1% Tween 80. At predetermined time points, 100 μL of release medium was sampled with replacement to which 3 mL of scintillation cocktail was added and vortexed before liquid scintillation counting. The cumulative amount of paclitaxel released as a function of time was calculated. In addition, to simulate the in vivo conditions, release of paclitaxel in presence of lysozyme (2 mg/100 mL) was also studied.

Film Thickness

Film thickness was measured using a micrometer (Mitutoyo, Kanagawa, Japan) with the smallest possible unit measurement count of 0.01 mm.

Tensile Strength

The effect of paclitaxel on mechanical properties of chitosan films was assessed through a tensile strength test. Tensile

strength of film was measured using texture analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) with the following acquisition parameters:

1. 2 mm/s prespeed
2. 1 mm/s test-speed
3. 10 mm/s postspeed with an acquisition rate of 50 points/s
4. 5 kg load cell

Film was secured with tensile grips, and a trigger force of 5 g was applied. The resulting profiles were analyzed using Texture Expert, Version 1.22 (Stable Micro Systems, Surrey, UK).

Solid-State Characterization

To study the molecular properties of paclitaxel and chitosan, the solid-state characterization was done by the application of thermal, infrared, x-ray diffraction, and microscopy techniques. During these studies, solid-state characteristics of paclitaxel and chitosan were compared with those of film to reveal any changes occurring as a result of film preparation.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) studies were performed with a Mettler Toledo 821^e thermal analyzer (Greifensee, Switzerland) calibrated with indium as standard. For thermogram acquisition, sample sizes of 1 to 5 mg were scanned with a heating rate of 5°C/min over a temperature range of 25°C to 300°C. In order to check the reversibility of transition, samples were heated to a point just above the corresponding transition temperature, cooled to room temperature, and reheated up to 300°C.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectra were obtained for paclitaxel, chitosan, blank films, and paclitaxel films on Nicolet Impact 410 (Nicolet Analytical Instruments, Madison, WI). Spectra of paclitaxel and chitosan were obtained using the potassium bromide disc method, while those of films were acquired directly. In each case, 100 spectra in the region of 400 to 4000 cm⁻¹ were co-added with a resolution of 2 cm⁻¹.

Scanning Electron Microscopy

Paclitaxel samples and chitosan films were viewed using a Jeol scanning electron microscope (SEM), JSM 1600 (Tokyo, Japan) for morphological examination. Powder samples of paclitaxel and films were mounted onto aluminum stubs using double-sided adhesive tape and then sputter coated with a thin layer of gold at 10 Torr vacuum before exam-

ination (Jeol Fine Coat, ion sputter, JFC-1100). The specimens were scanned with an electron beam of 1.2 kV acceleration potential, and images were collected in secondary electron mode.

X-ray Diffraction Studies

Molecular arrangement of paclitaxel and chitosan in powder as well as in films was compared by powder x-ray diffraction patterns acquired at room temperature on a Philips PW 1729 diffractometer (Eindhoven, Netherlands) using Cu K α radiation. The data were collected over an angular range from 3° to 50° 2 θ in continuous mode using a step size of 0.02° 2 θ and step time of 5 seconds.

In Vivo Implantation Studies

Biodegradation of films was studied in Swiss mice. Initially, mice were anesthetized with thiopentone sodium (40 mg/kg) and occasional light ether inhalation, and an incision was made in the back of the neck region with a scalpel. After incision, the implantation site was created by tunneling immediately beneath the skin, then films were inserted and the skin was sutured. To prevent infection, mice were given gentamicin (2 mg/kg, intraperitoneal route) every 4 days. For in vivo implantation purposes, film was prepared in a plastic mold of radius 1.15 cm (instead of glass plate) with each mouse receiving one such film.

Histology Studies

Histology studies were performed to examine the acute toxicity of film at the implantation site. After a 2-month implantation period, mice were humanely killed by cervical dislocation and an incision was made in the implantation area. Then, the tissue in which the film was imbibed was removed and stored in 50% formalin until processing. Subsequently, tissue processing involved dehydration through a graded series of alcohols (70%, 80%, 95%, and 100%), followed by xylene and then infiltration with paraffin. For obtaining thin sections (3-5 μ m), tissues were embedded on the edge of paraffin blocks and were cut on a rotary microtome. These sections were deparaffinized, rehydrated with graded alcohols (100%, 95%, 80%, and 75%), and stained with hemotoxylin/eosin for microscopic examination.¹⁸ Similarly, sections of paclitaxel chitosan film and tissue of healthy mouse were obtained to serve as control.

RESULTS

Preparation of Films

The difficulty of incorporating water-insoluble paclitaxel molecules was circumvented by concomitant inclusion of unsatu-

Table 1. Composition and Thickness of Paclitaxel Chitosan Films Obtained by Casting Method*

Film Code	Chitosan (mg) [†]	Glycerol (mg)	Phosphatidyl Choline (mg) [‡]	Phosphatidyl Glycerol (mg) [‡]	Poloxamer 407 (mg) [§]	Paclitaxel (mg)	Film Thickness (µm)
FLM-1-PCL	200	100	130	15	-	10	50-60
FLM-2-PCL	200	100	130	15	-	15	50-60
FLM-3-PCL	200	100	130	15	-	20	50-60
FLM-4-PCL	150	100	-	-	50	10	40-45
FLM-5-PCL	25	10	-	-	10	20	100-120

*FLM indicates film. Films 1, 2, 3, and 4 were cast on glass plate of surface area 45.5 cm², while film 5 was cast in plastic mold of surface area 4.2 cm². Films 1, 2, and 3 were dried at 37°C; films 4 and 5 were dried at 60°C.

[†]Chitosan is ≥85% deacetylated.

[‡]Liposomes containing paclitaxel were prepared from phospholipids of soya origin by film hydration method and used without extrusion; encapsulation efficiency ~95%.

[§]The type of poloxamer used here could be replaced with other grades or other polymers which might lead to better formulation.

^{||}Formulation was selected for physicochemical characterization and histology studies (see text).

rated phospholipids (in the form of liposomes) or poloxamer along with paclitaxel in film (Table 1). Unsaturated lipids were used to prepare liposomes (as a means of incorporation of paclitaxel in film) since earlier studies have shown that these lipids yield best encapsulation efficiency of paclitaxel (D.A. and R.P., unpublished data, 2000). These films were obtained by casting method and appeared either transparent or translucent and were pale yellowish in color. Further, poloxamer-containing films showed good loading capacities of 20 mg of paclitaxel per 25 mg of chitosan, and these films had a mean weight of 63.5 mg. At this loading percentage (30%-31%), essentially all drug could be incorporated into film without any precipitation. A lower fabrication temperature of 37°C was chosen for lipid films in contrast to poloxamer films (60°C) in order to minimize hydrolysis and oxidation of unsaturated phospholipids. When films were examined for thickness, the lipid films (FLM-1,2,3-PCL) ranged from 50 to 60 µm, while poloxamer films FLM-4-PCL (cast on glass plate) ranged from 40 to 45 µm, and FLM-5-PCL (cast in plastic mold) ranged from 100 to 120 µm. At constant casting surface area, the higher thickness of lipid films (FLM-1,2,3-PCL) over poloxamer films (FLM-4-PCL) is due to phospholipids and a higher amount of chitosan. Although initial attempts to incorporate paclitaxel with phospholipids were found to be feasible, since unsaturated lipids are prone to oxidation, only chitosan-poloxamer films containing paclitaxel were chosen for further characterization unless specified.

Chemical Stability of Paclitaxel

In the present study, films were prepared by the classical method, which involves spreading a uniform layer of polymer dispersion followed by a drying step for removal of solvent system. Since film preparation methodology involved a heating step, it may have had a detrimental effect on the chemical stability of drug. Hence, stability assessment of paclitaxel impregnated in film was done using stability-indicating

method. For this purpose, paclitaxel was extracted from film and analyzed by HPLC. A single peak at 18.5 minutes representing paclitaxel (with no additional peaks) was detected in the chromatogram, suggesting that the molecule was stable during preparation of films (chromatograms not shown).

Content Uniformity

Paclitaxel was extracted from different regions of chitosan film using acetonitrile:ETOH (1:1 vol/vol) solvent system. After normalization of amount of paclitaxel on weight basis of film, the results indicated that the variation in distribution of paclitaxel in different regions of film was <15% (results not shown).

Release Studies

In order to establish the ability of films to serve as depot formulations, release of paclitaxel from both lipid- and poloxamer-containing films was studied. It was observed that release was negligible from lipid-containing films, while those containing poloxamer showed a burst effect followed by no release. Films containing poloxamer released ~10% in 6 hours; further release of paclitaxel was not observed until the study period of 144 hours, suggesting that the film had retained 90% of the payload (Figure 1A). Since the release of paclitaxel was <10%, further studies were undertaken in presence of lysozyme (chitosan is a substrate for the enzyme) to simulate the in vivo conditions. However, no significant difference was observed in presence of lysozyme (Figure 1B), suggesting that this model was not appropriate to simulate in vivo conditions for release-rate studies.

Mechanical Strength of Film

Mechanical strength of film is described in terms of tensile strength, and brittle films are characterized by a decrease in

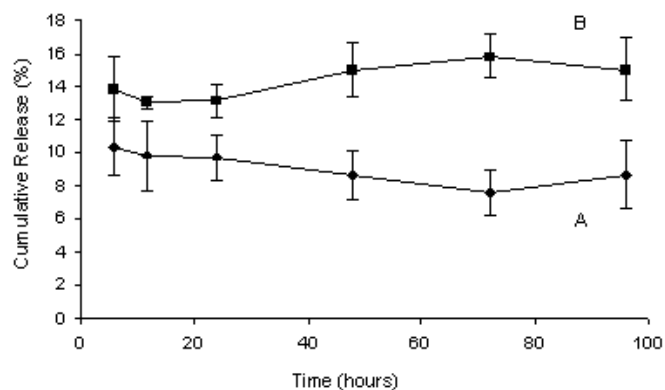


Figure 1. Cumulative percentage release (in vitro) of paclitaxel from chitosan film at 37°C in (A) absence and (B) presence of lysozyme (2 mg/100mL); 0.1% Tween 80 was used in release medium to provide sink conditions (data are mean \pm SD; n = 3).

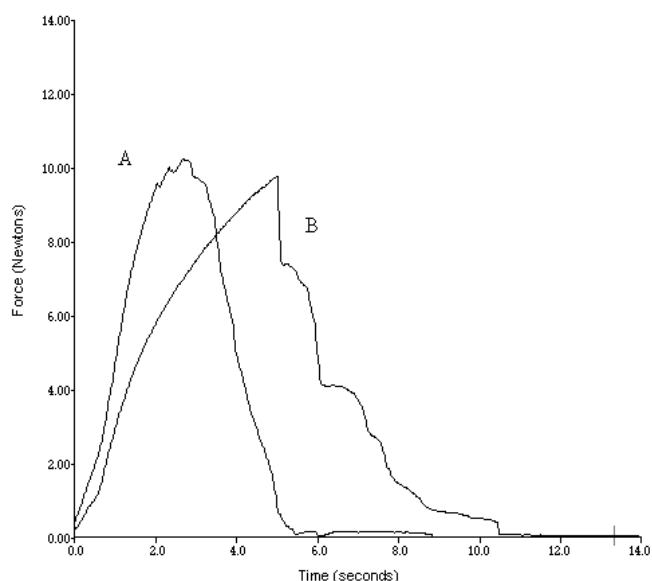


Figure 2. Force-time profiles of (A) blank film and (B) paclitaxel chitosan film to study the influence of formulation approach on mechanical properties of film.

the percentage of elongation at break. The area under curve is related to the energy required to break polymeric material, and tough polymers have larger areas requiring large amounts of energy for rupture. In order to understand the arrangement of polymer chains in the presence of paclitaxel, force-time profiles of films were generated as shown in Figure 2. Although force of elongation at break is slightly lowered in presence of paclitaxel in film (10.3 vs 9.8 N), the area under the profile has been increased (31.3 vs 45.9 N). For convenience of interpretation, each profile is further described in terms of ascending and descending portions. The time to plateau of the ascending portion of paclitaxel-chitosan film was greater in comparison with control film. As brittleness is reflected in the time to plateau, greater time is

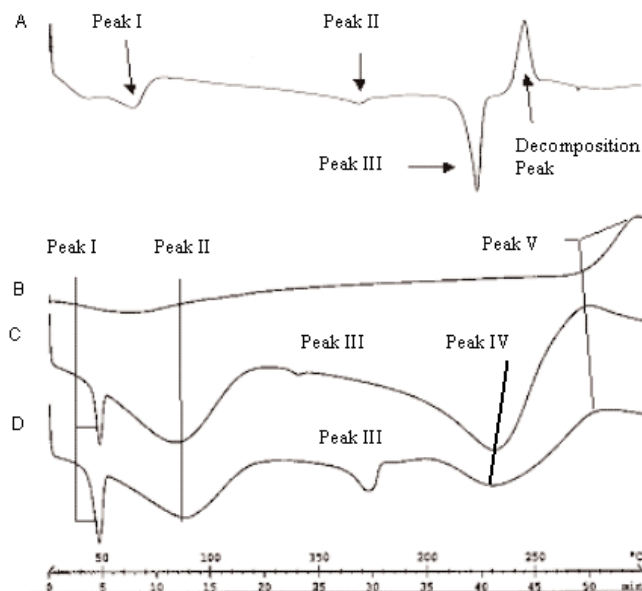


Figure 3. DSC studies to investigate physical transformations induced in chitosan and paclitaxel by comparing solid-state features of pure components with that in films (A) paclitaxel, (B) chitosan powder, (C) blank film, and (D) paclitaxel chitosan film. Thermograms were obtained at a scan rate of 5°C/min. When paclitaxel chitosan film was heated to 190°C, cooled to 25°C, and reheated, peaks I, II, and III were found to be irreversible in nature.

indicative of lack of brittleness of film. In addition, the descending segment of the profile of control film was uniform, while that of paclitaxel film was irregular and protracted. The discontinuities in internal structure and variation in strength of film matrix may be the cause of the irregular descending portion of the profile.

Solid-state Characterization

Thermal Studies of Films

The DSC thermograms of paclitaxel, recrystallized paclitaxel, blank, and paclitaxel-chitosan films are shown in Figure 3, and the observed thermal events are summarized in Table 2. Thermogram of paclitaxel showed an initial broad peak at 64.5°C (Peak I, Figure 3A) due to removal of absorbed moisture or nonstructural water followed by a single endotherm at 223.6°C (Peak III, Figure 3A) just prior to an exotherm of degradation peak. Another minor broad peak at 168.9°C (Peak II, Figure 3A) was observed, which is due to the presence of small amounts of paclitaxel dihydrate in the sample¹⁹ (the peak was absent from second heating phase on DSC run when the sample was initially heated to 200°C, cooled back to 25°C, and reheated; results not shown). Liggins et al¹⁹ have previously ascribed this peak to solid-solid transition associated with the conversion of dehydrated paclitaxel dihydrate to semicrystalline form. DSC studies were also performed on dry powder (recrystallized paclitaxel) obtained by evapora-

Table 2. Details of Thermal Events Obtained by Differential Scanning Calorimetry for Paclitaxel, Recrystallized Paclitaxel, Chitosan Powder, Blank Chitosan Film, and Paclitaxel Chitosan Film*

Sample	Peak I		Peak II		Peak III		Peak IV		Peak V
	T	ΔH	T	ΔH	T	ΔH	T	ΔH	T
Paclitaxel	64.5	22.24	168.9	17.17	223.6	54.69	-	-	241.1
Recrystallized paclitaxel	38.1	4.54	49.7	6.29	63.2	5.81	197.4	2.19	219.6
Chitosan powder	68.2	145.63	-	-	-	-	-	-	294.5
FLM-5-BLK	48.3	6.93	85.3	78.99	139.9	0.72	230.4	129.22	274.5
FLM-5-PCL	48.1	9.46	88.5	92.65	172.5	15.28	230.9	36.65	280.1

*T indicates peak temperature in °C; and ΔH , enthalpy of peak in J/g. Peaks I, II, III, and IV are endothermic, while peak V represents exothermic events. Peaks I, II, III, and IV are due to transitions/melting, while peak V is a result of decomposition. Peak number of one sample has no relevance with peak number of other samples. Peaks I, II, and III are irreversible events for film samples. Decomposition peak of chitosan was considerably broadened on casting into film. Note that although SEM photomicrographs of recrystallized paclitaxel and paclitaxel film reveal the presence of microparticles, the corresponding melting endotherms were not observed here, probably owing to lack of sensitivity of DSC to detect the minute percentage of the same in samples.

tion of paclitaxel-ploxamer mixtures dispersed in aqueous acetic acid-ETOH solvent system (with same proportions as mentioned in Table 1). The only differences were that chitosan and glycerol were omitted. In DSC, thermograms of recrystallized paclitaxel (not shown), a single endotherm of transition of ploxamer at 49.7°C (Peak II, Table 2), and 2 minor transitions were observed. However, a transition peak of paclitaxel with very low heat of fusion was seen at 197.4°C (Peak IV, Table 2), while the melting endotherm at 223.6°C was absent. On the other hand, the degradation peak of paclitaxel appeared at 219.6°C (Peak V, Table 2).

Both blank and paclitaxel-chitosan films exhibited 4 endothermic peaks in DSC thermograms. The transition of ploxamer in these films occurred at 48°C (Peak I, Figures 3C and D) with no appreciable shift. Other peaks in the region of 85°C to 88°C (Peak II) have resulted from loss of moisture on heating. In paclitaxel-loaded film, an asymmetric peak representing thermal event of a new form of paclitaxel was seen at 172.5°C (Peak III, Figure 3D). This event was followed by a broad endothermic peak at 230°C to 240°C (Peak V, Figures 3C and D) in both films, which may be attributed to glycerol component. However, thermal events of paclitaxel, namely, melting and decomposition, which were previously noted in the region of 190°C to 225°C were not observable in film (Figure 3D). Further, decomposition of chitosan was observed as a broad exotherm in films at 274°C to 280°C, while the same decomposition exotherm was at 294.5°C (Peak V, Figure 3B) for chitosan powder under identical experimental DSC conditions.

Fourier Transform Infrared Spectroscopy

Transmission infrared spectra of chitosan powder, paclitaxel, and films were acquired to draw information on the molecular state of chitosan and paclitaxel (Figure 4A). Chitosan is an amino glucose characterized by a small proportion of amide groups via an amide linkage with acetic acid. In the infrared spectrum, powder chitosan exhibited a broad peak at

3431 cm^{-1} , which is assigned to the N-H and hydrogen bonded O-H stretch vibrational frequencies, while a sharp (shoulder) peak at 3610 cm^{-1} is that of free O-H bond stretch of glucopyranose units. Further, in the C-H stretch region of FTIR spectrum, the higher intensity peak at 2923 cm^{-1} is assigned to the asymmetric and the lower intensity peak at 2857 cm^{-1} is assigned to the symmetric modes of CH_2 . In addition, the characteristic band due to CH_2 scissoring, which usually occurs at 1465 cm^{-1} was also present in the sample. Since the grade of chitosan used in the present study was $\geq 85\%$ deacetylated, an amide bond peak was present in the spectra and the C=O stretch of amide bond was observed at 1661 cm^{-1} . The peaks at 1550 and 1599 cm^{-1} were assigned to strong N-H bending vibrations of secondary amide, which usually occur in the range of 1640 to 1550 cm^{-1} as strong band.²⁰

In comparison to the chitosan powder, spectrum was not sharp in film. An overlay of chitosan film and paclitaxel-chitosan film is shown in Figures 4B and C. The presence of residual moisture content and glycerol in films resulted in a broad peak from 3500 to 2800 cm^{-1} . The peak at 1651 cm^{-1} , representative of C=O stretch of amide bond in chitosan film, shifted to 1648 cm^{-1} in the presence of paclitaxel. Further, the peak due to N-H bending vibration, which was observed at 1588.6 cm^{-1} in chitosan film, was lowered to 1579 cm^{-1} in paclitaxel-chitosan film; its intensity also decreased. The band due to CH_2 scissoring, which occurred at 1466 cm^{-1} , was broadened due to paclitaxel in film. However, only 2 characteristic peaks of paclitaxel, at 1740 and 1706 cm^{-1} , were observed in paclitaxel-chitosan film, while other peaks were not discernible due to interference caused by polymers.

In order to abstract spectral features of paclitaxel, infrared spectra of control chitosan film were subtracted from that of paclitaxel-chitosan film as shown in Figure 4E. A clear loss in resolution of infrared features of paclitaxel in film was observed when compared with that of powder paclitaxel (Figure 4D). The peak due to C=O amide stretch at 1646 cm^{-1}

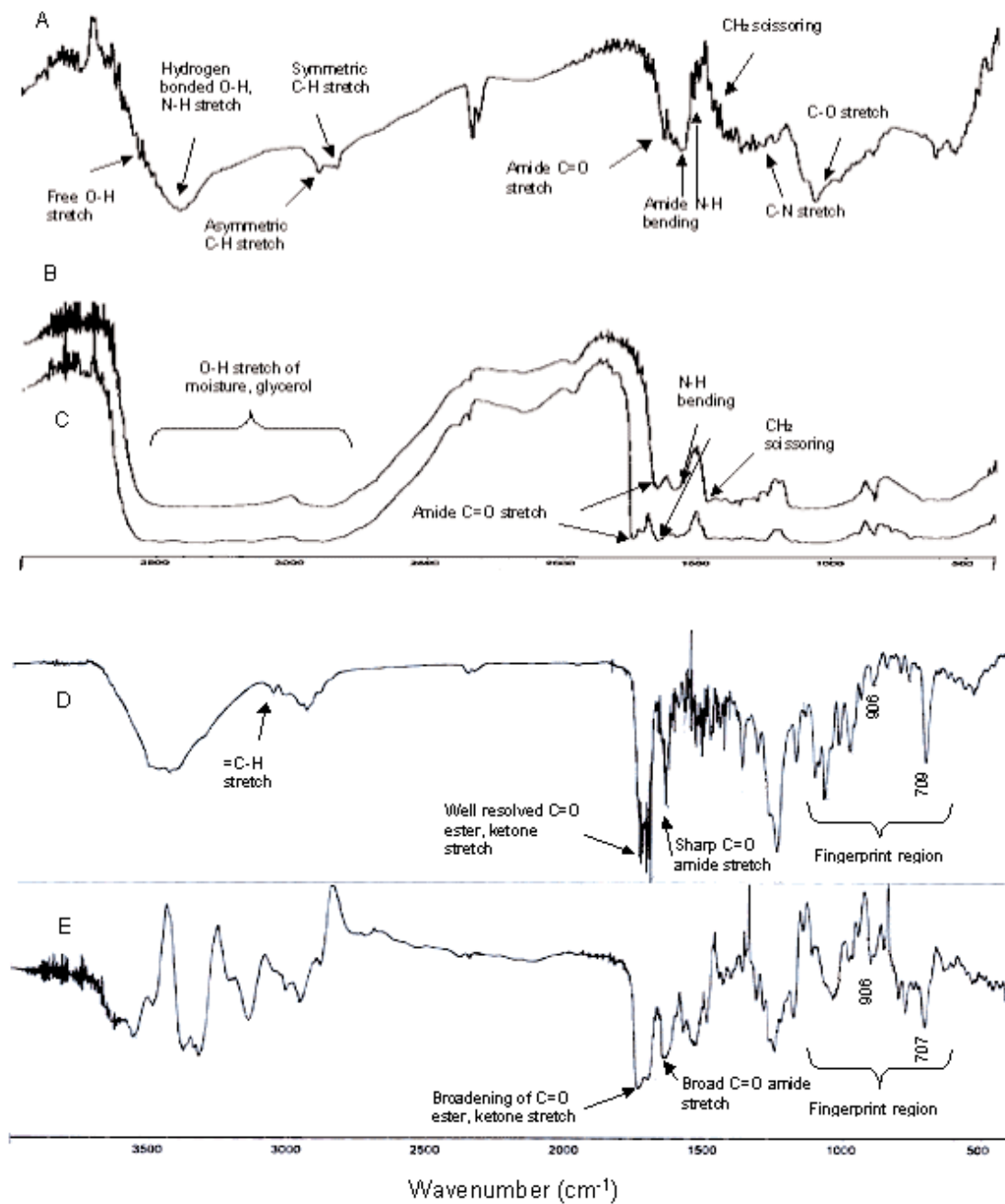


Figure 4. FTIR spectra of (A) chitosan powder, (B) blank film, (C) paclitaxel chitosan film, (D) paclitaxel, and (E) subtraction result of blank film from paclitaxel chitosan film to examine the alterations in molecular vibrations of paclitaxel and chitosan induced as a result of film fabrication. All spectra are plotted in transmittance mode.

considerably broadened in film in comparison with powder paclitaxel. Further, the peak representing CH₂ scissoring mode of paclitaxel at 1451 cm⁻¹ decreased in intensity in film. A shift in wave number owing to CH₃ bending at 1370 cm⁻¹ of paclitaxel to 1366 cm⁻¹ occurred.

Film Morphology Studies

SEM photomicrographs revealed that commercial sample of paclitaxel has a plate-like appearance, while recrystallized sample appear fibrous and elongated, as shown in Figures 5A and

B. In addition, SEM photomicrographs of control and paclitaxel films were acquired and compared with that of paclitaxel samples. The morphology of control film was plain and the picture appeared dark, indicating that the control film has a smooth surface (Figure 5C). In contrast to control film, features of paclitaxel-chitosan film resembled that of fibrous recrystallized paclitaxel morphology (Figure 5D). This typical surface appearance suggests that paclitaxel is not only dispersed in the film matrix but also projected onto the surface of film. Some irregularly shaped particles were also identified in the pictures of recrystallized paclitaxel and film loaded with paclitaxel.

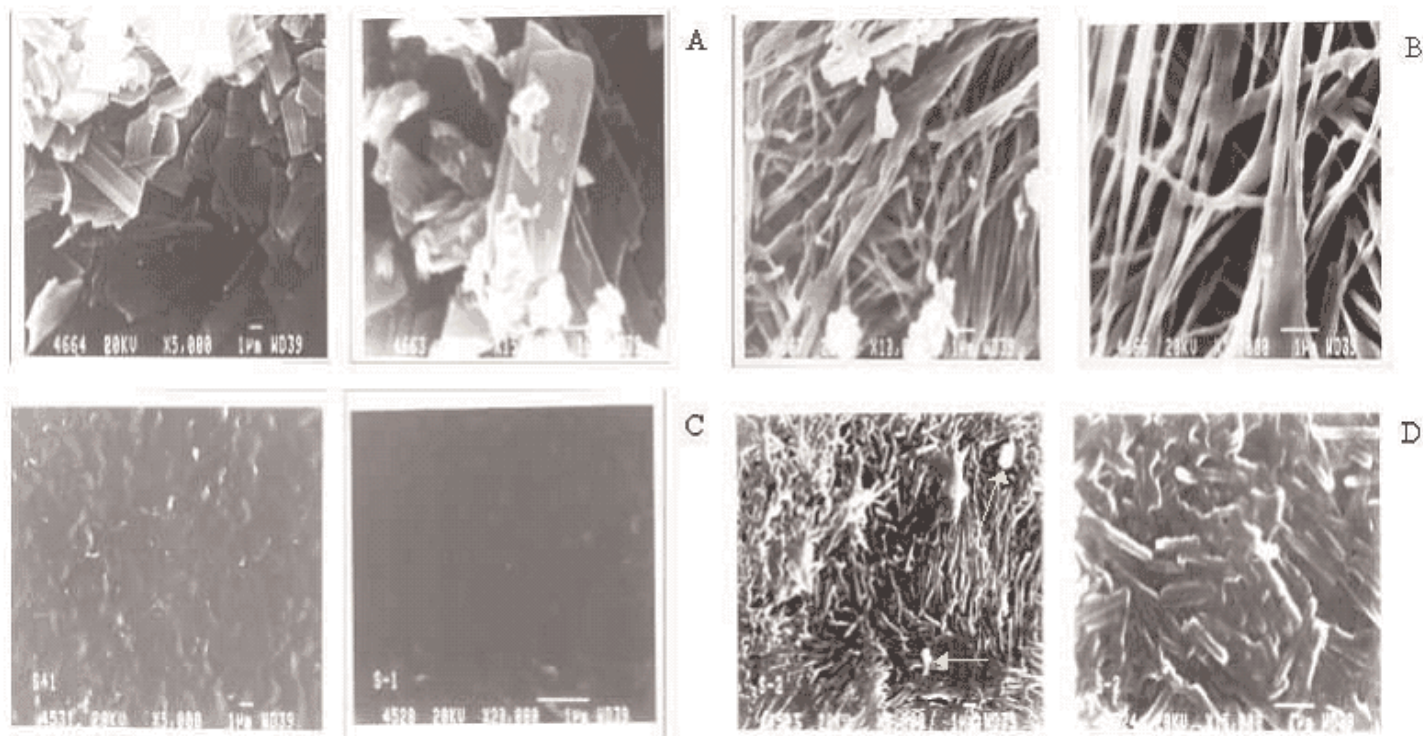


Figure 5. Scanning electron photomicrographs of (A) paclitaxel, (B) recrystallized paclitaxel, (C) blank film, and (D) paclitaxel chitosan film obtained at different magnifications. Paclitaxel photomicrographs were that of commercial sample without any modification. Recrystallized paclitaxel was obtained by evaporation of hydro-ethanolic solution of paclitaxel and poloxamer 407 under identical conditions as used for film casting in the absence of chitosan (see Table 1). Magnification: (A) $\times 5000$ (left); $\times 15\,000$ (right); (B) $\times 10\,000$ (left); $\times 15\,000$ (right); (C) $\times 5000$ (left); $\times 20\,000$ (right); (D) $\times 5000$ (left); $\times 15\,000$ (right). The arrows in (D) indicate the presence of poloxamer coated paclitaxel microparticles.

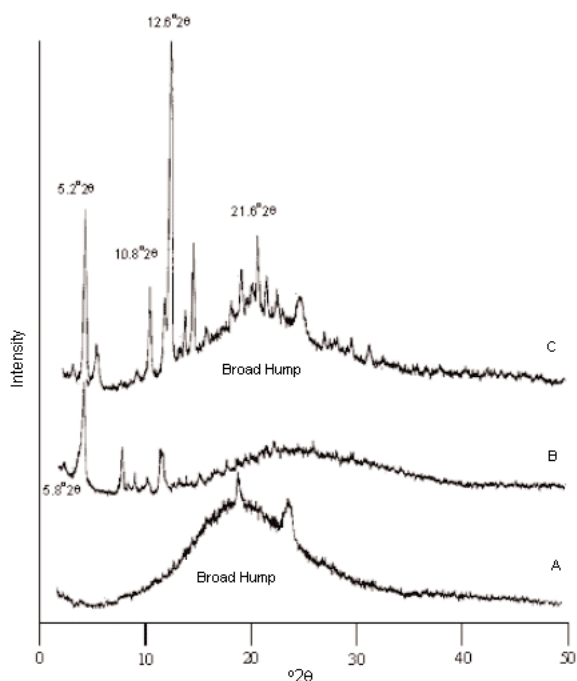


Figure 6. X-ray diffraction patterns of (A) blank chitosan film, (B) paclitaxel, and (C) paclitaxel chitosan film at ambient temperature to examine solid-state features of paclitaxel in film (the overlay picture is obtained by tracing out individual patterns by hand).

X-ray Diffraction Studies

X-ray diffraction is a proven tool to study crystal lattice arrangements and yields very useful information on degree of sample crystallinity. X-ray diffraction pattern of paclitaxel, blank, and paclitaxel film were obtained and compared, which revealed marked differences in the molecular state of paclitaxel (Figure 6). The diffractogram of blank chitosan film has shown 2 low intensity peaks at 19.1° and 23.3° 2θ with a characteristic broad hump in the range of 7° to 45° 2θ . This halo diffraction pattern (broad hump) is an indication of the predominantly amorphous form of chitosan in films (Figure 6A). In the case of paclitaxel, the diffractogram exhibited peaks at the following 2θ values: 4.1° , 5.4° , 5.8° , 9.1° , 10.2° , 11.3° , 12.3° , and 12.5° (Figure 6B). Among these, the peak of highest intensity was located at 5.8° 2θ , and the peaks at 11.3° and 12.5° 2θ were broad. When the diffraction pattern of paclitaxel in chitosan film was compared with that of paclitaxel, the pattern differed to a large extent. Several high-angle diffraction peaks were observed in paclitaxel-chitosan film at the following 2θ values: 4.1° , 5.2° , 6.2° , 10.8° , 12.1° , 12.6° , 13.8° , 14.6° , 18.6° , 20° , 20.7° , 21.6° , and 23.6° (Figure 6C). The 12.6° 2θ peak had the highest intensity, and the hump in the baseline occurred from 7° to 45° 2θ , as observed for chitosan film.

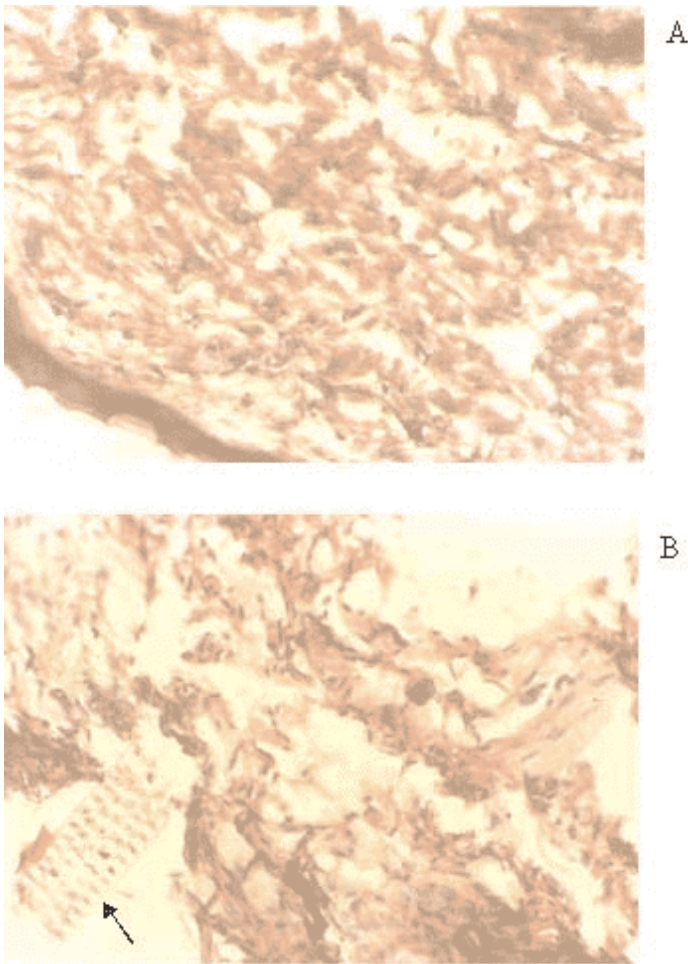


Figure 7. Histopathological examination for generalized inflammatory response to assess biocompatibility of subcutaneous implanted chitosan film in mice (A) control tissue and (B) paclitaxel chitosan film embedded tissue following implantation of the film after 60 days (magnification $\times 250$). Film is indicated by arrow in (B). Histological assessment is usually based on cellular infiltration between and around delivery system, edema, formation of granulation tissue, and presence of multinucleated giant cells/macrophages.

It is probable that the new sharp diffraction peaks are the result of a new form of paclitaxel.

In Vivo Implantation Studies of Films

In order to confirm in vivo degradation of film, a time-dependent visual monitoring of film texture and integrity following implantation in Swiss mice was performed. On days 2, 10, 20, and 50, mice were humanely killed by cervical dislocation, and an incision was made in the site for examination of film. After 2 days of implantation, it was observed that (though film was intact) film turned white and opaque when compared with the control film, which was initially yellowish and translucent. With progression of implantation period, film turned soft and delicate (on the 10th day) with loss of mechanical strength. In a period of 30 to 40 days, film commenced to get embed into

surrounding tissues, and after 50 days it was completely embedded. Although this experiment was qualitative in nature, the film's loss of integrity and identity over time confirms that the implant was amenable to biodegradation.

Histology Studies

The tissue responses to implanted film were studied by microscopic examination of tissues in the implanted area (Figure 7). Lack of an increase in macrophages at the site of implantation suggests that inflammatory responses were either minimal or absent.²¹ It has been reported previously that the degree of deacetylation of chitosan is a key factor in determining not only release rate but also inflammatory responses.²² Typically, chitosan gels with low degrees of deacetylation have short residence times and higher inflammatory response, while those with higher degrees have longer residence time and minimal or no inflammation.²² In the present study, the grade of chitosan used for fabrication of film was $\geq 85\%$ deacetylated; hence, the high degree of deacetylation of chitosan may be the reason for reducing local inflammatory responses following implantation.

DISCUSSION

In order to develop a local delivery system for paclitaxel, a biodegradable chitosan film was attempted, which, to our knowledge is the first effort of its kind. Potential formulation problems were anticipated since chitosan is only soluble in aqueous acidic solutions, whereas paclitaxel, being a hydrophobic drug, is insoluble under similar conditions. In early stages of formula optimization studies, it was observed that paclitaxel was not incorporated into film but crystallized out as needles owing to lack of solubilization in aqueous chitosan solutions. Hence, initial attempts were directed toward the use of liposomes as carriers. Despite the success, lipids were subsequently replaced with poloxamer since lipids are prone to oxidation and hydrolytic degradation processes, which are further accelerated under heat.²³⁻²⁴ In this regard, poloxamer was found to be an attractive alternative since crystallization of paclitaxel (as needles, which was observed during our initial attempts) did not occur with this formulation strategy. The exact mechanism by which the incompatibility is obliterated is not known, but it is plausible that poloxamer micelles act by a solubilization phenomenon that is further stabilized by ETOH.

Under in vitro conditions, poloxamer film exhibited an initial burst effect followed by negligible release of paclitaxel (Figure 1). The initial burst release from the films is attributed to the surface-deposited paclitaxel as revealed by SEM photomicrographs (Figure 5), while the subsequent lack of release might be due to non-erosion/-degradation of film. As chitosan is insoluble in the release medium used for the study,

the film failed to release drug, and this trend was observed with all film preparations. However, the extraction procedure adopted to study content uniformity of drug in films showed a 100% recovery into medium that is composed of acetonitrile:ETOH (1:1 vol/vol). In spite of insolubility of chitosan in the above extraction medium, the complete and high recoveries imply that the pores available within film are large enough to allow diffusion of paclitaxel (854 Da) into the medium, which possesses sufficient solubility for this hydrophobic molecule. Although the test medium contained Tween 80, release of paclitaxel was not observed since the resulting micelles (diameter of 20–30 nm) were probably inaccessible to the inner matrix of film. Nevertheless, it is worth mentioning that release from film was compared with that of liquid extraction (with organic solvent) only to elucidate the behavior of film at the molecular level and its effect on liberation of incorporated paclitaxel. Although the organic phase may represent a suitable medium for release rate studies of paclitaxel, such studies were not conducted because they have no biological relevance.

To simulate the biodegradation process, lysozyme was included in the release medium because chitosan is susceptible to degradation by this enzyme.³ The extent of polymer degradation by lysozyme was observed to be inversely proportional to the degree of deacetylation and molecular weight.²⁵ However, presence of lysozyme did not significantly influence the release of paclitaxel from films (Figure 1). In similar studies, chitosan microspheres were not degraded by the enzyme for up to 20 days.¹⁰

The primary mechanisms for release of drugs from matrix systems *in vitro* are swelling, diffusion, and disintegration,²⁶ while *in vivo* release is governed by both diffusion and biodegradation of matrix. Tomihata and Ikada²⁷ reported that the *in vitro* and *in vivo* degradation of chitosan films prepared by solution casting method occurred less rapidly as the degree of deacetylation increased, and films that were greater than 73% deacetylated showed slower biodegradation. Since the grade of chitosan used in the present study was of high molecular weight with a degree of deacetylation $\geq 85\%$, significant retardation of release of paclitaxel from film is attributed to the polymer characteristics. In addition, diffusion of paclitaxel may have been hindered by increased tortuosity of polymer accompanied by a swelling mechanism.

Films exhibited sufficient mechanical strength when assessed in terms of the force of breaking point and percentage elongation at break. In general, stiff and brittle materials display a steep rise in stress-strain curve (undergoing little or no cold flow), which extends only over narrow areas, indicating that it doesn't take much force to break them and that they have low impact resistance. It was also observed that paclitaxel film ruptures gradually rather than abruptly (an extended profile) on application of force, leading to increased area under

stress curve and imparting toughness to the film. The elongated fibrous structures in paclitaxel-chitosan film, revealed by SEM (Figure 5) form an entangled network with chitosan polymer chains during film formation, thereby, altering the film's elongation at break. These observations indicate that chitosan films are not brittle in nature but tough and flexible, and discontinuities in film are caused by interruptions in film matrix by fibrous poloxamer-paclitaxel structures.²⁸

As DSC is a useful tool to monitor the effects of additives on the thermal behavior of materials, this technique was used to derive qualitative information about the physicochemical status of drug in films.²⁹ The recrystallized paclitaxel showed 2 peaks, a low endotherm peak representative of transition temperature of poloxamer and an exothermic peak for degradation of paclitaxel. The absence of a second endothermic peak in the DSC run above 200°C for recrystallized paclitaxel-poloxamer mixture suggests that paclitaxel was present in an amorphous form (Table 2). Amorphous paclitaxel prepared by quench cool method was reported to have a glass transition temperature of 152.4°C with no characteristic melting peak of paclitaxel.¹⁹ A new peak at 172.5°C (which was not present in control films) was observed in paclitaxel-chitosan films (Figure 3). The melting/transition point, as well as heat of transition of this peak, was different and higher than that of amorphous glass transition, suggesting that the peak arises due to melting/transition of a new solid-state form of paclitaxel. Further, samples were subjected to a heat-cool cycle (25°C-190°C-25°C-300°C) in order to ascertain the nature of this thermal event. On the second heating phase of heat-cool cycle, all peaks were absent from the recrystallized sample as well as from paclitaxel-loaded film, suggesting that these events are irreversible and that the peak at 172.5°C (in film) represents a transition and not a melting process. In addition, the decomposition peak of chitosan shifted to lower temperatures in film, which may indicate loss of interpolymer chain interactions as chitosan films were reported to undergo thermal decomposition at 290°C to 300°C.³⁰

In order to impart both plasticizing and dispersing properties, glycerol and poloxamer were included in film. In addition to improving solubility of paclitaxel, these components resulted in loss in prominence of infrared features owing to severe background absorbance, which complicated the interpretation of spectra. Three basic infrared features of chitosan powder, namely the amide C=O stretch, amide N-H bend, and CH₂ scissoring modes, are compared with that of film. The C=O stretching frequency of amide was found to be lower in both the films, with the shift being greater in presence of paclitaxel. This shift is attributed to hydrogen bonding with hydroxyl groups of glycerol,³¹ whereas shifts in N-H bending and CH₂ scissoring frequencies indicate altered interaction/mobility of polymer chains. At the same time, infrared spectra of paclitaxel in film were also compared with that of

pure form, and differences were observed in spectral features from 3600 to 1500 cm^{-1} and in the fingerprint region of paclitaxel. These changes reflect the differences in intermolecular interactions between commercial paclitaxel and paclitaxel incorporated in chitosan film, which arise owing to solid-state modification. These differences include broadening of C=O bond, decreased intensity of CH_2 scissoring mode, and lowering of CH_3 bending frequency, suggesting that paclitaxel has an altered degree of crystallinity in film (Figure 4).

As observed from SEM photomicrographs, the crystals of paclitaxel (plate-like) have a different appearance than recrystallized paclitaxel (elongated and fibrous) (Figure 5). This dissimilarity suggests that the crystal habit and/or lattice of paclitaxel is modified during film casting. In addition, the photomicrographs of recrystallized paclitaxel and paclitaxel-loaded film showed scattered irregularly shaped microparticles. It is reasoned that the proportion of poloxamer was not sufficient to form dispersion with the total amount of paclitaxel, and the excess paclitaxel was observed to precipitate as irregularly shaped particles. However, these microparticles do not have clearly defined crystal morphological features in the SEM photomicrographs. Hence, it appears that the irregularly shaped particles are surface deposited with poloxamer, which gives them an appearance resembling that of coated particles. However, melting peak for the poloxamer-coated crystalline form of paclitaxel was not observed in DSC curves, probably owing to the small percentage and the insensitivity of the instrument to record the thermal event.

X-ray diffraction is also used to study the degree of crystallinity of pharmaceutical drugs and excipients. A lower 2θ value indicates larger d-spacings, while an increase in the number of high-angle reflections indicates higher molecular state order. In addition, broadness of reflections, high noise, and low peak intensities are characteristic of a poorly crystalline material. A broad hump in the diffraction pattern of chitosan extending over a large range of 2θ suggests that chitosan is present in amorphous state in the film (Figure 6A). The 2 broad peaks superimposed on the hump, also observed in other studies, were attributed to hydrated and anhydrous crystals of chitosan in film.³⁰ Film of paclitaxel has exhibited a greater number of peaks than paclitaxel, and, shifts were observed for most of the peaks, with the most intense peak located at higher 2θ value in film (Figure 6C). The dissimilarity in diffraction pattern is indicative of a different crystalline form of paclitaxel in film, while the more intense and several new diffraction peaks provide evidence of increased crystallinity of paclitaxel. The high-angle diffraction peaks are probably due to the thermally annealed dehydrated/desolvated polymorph produced during the drying process of film formation, representing the polymorphic transition peak at 172.5°C in DSC thermogram. Hence, x-ray diffraction studies and SEM photomicrographs show that

film casting methodology converted paclitaxel into a new crystalline form with a different crystal lattice arrangement than the commercial sample (paclitaxel).

Although chitosan film retained its integrity in the presence of lysozyme *in vitro*, it has demonstrated susceptibility to biodegradation under *in vivo* conditions in mice. Moreover, tissue examined at the site of implantation did not show any significant infiltration of macrophages, signifying that film had not imposed any unfavorable influences to create hostile environment. These studies demonstrated that normal physiological processes in host tissue remain unaffected after implantation of film.

CONCLUSION

Chitosan films containing paclitaxel were obtained by casting method with high loading efficiencies, and the chemical integrity of molecule was unaltered during preparation. Following an initial burst release, paclitaxel, being a hydrophobic drug, was not released further from the high molecular weight chitosan films. Since films were observed to be susceptible to *in vivo* biodegradation mechanisms, paclitaxel release is expected in an appropriate environment that causes disruption of film either in the form of erosion or degradation *in vivo* to facilitate release of the hydrophobic molecule. Paclitaxel exhibits dose-dependent antiangiogenic, antimetastatic, and apoptotic properties to block multiple pathways involved in the growth and spread of cancer. Hence, it is anticipated that this delivery system would successfully allow simultaneous harnessing of therapeutic attributes of the molecule by elevating "tumor burden" of the drug to improve quality of treatment and increase survival of cancer patients.

REFERENCES

1. Angelova N, Hunkeler D. Rationalizing the design of polymeric biomaterials. *Trends Biotechnol.* 1999;17:409-421.
2. Pillai O, Panchagnula R. Polymers in drug delivery. *Curr Opin Chem Biol.* 2001;5:447-451.
3. Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. *Biomaterials.* 1982;3:105-108.
4. Janes KA, Fresneau MP, Marazuela A, et al. Chitosan nanoparticles as delivery systems for doxorubicin. *J Control Release.* 2001;73:255-267.
5. Ruel-Gariepy E, Chenite A, Chaput C, et al. Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. *Int J Pharm.* 2000;203:89-98.
6. Muzarelli R, Baldassarre V, Conti F, et al. Biological activity of chitosan: ultra structural study. *Biomaterials.* 1998;9:247-252.
7. Senel S, Ikin G, Kas S, et al. Chitosan films and hydrogels of cholehexidine gluconate for oral mucosal delivery. *Int J Pharm.* 2000;193:197-203.
8. Ouchi T, Banba T, Fujimoto M, et al. Synthesis and antitumor activity of chitosan carrying 5-fluorouracil. *Makromol Chem.* 1989;190:1817-1825.

9. Jameela SR, Jayakrisnan A. Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: studies on the in vitro release of mitoxantrone and in vivo degradation of microspheres in rat muscle. *Biomaterials*. 1995;16:769-775.
10. Blanco MD, Gomez C, Olmo R, et al. Chitosan microspheres in PLG films as devices for cytarabine release. *Int J Pharm*. 2000;202:29-39.
11. Miwa A, Ishibe A, Nakano M, et al. Development of novel chitosan derivatives as micellar carriers of taxol. *Pharm Res*. 1998;15:1844-1850.
12. Ma J, Wang H, He B, et al. A preliminary in vitro study on the fabrication and tissue engineering applications of a novel chitosan bilayer material as a scaffold of human neonatal dermal fibroblasts. *Biomaterials*. 2001;22:331-336.
13. Miyazaki S, Yamaguchi H, Takada M, et al. Pharmaceutical application of biomedical polymers. XXIX. Preliminary study on film dosage form prepared from chitosan for oral drug delivery. *Acta Pharm Nord*. 1990;2:401-406.
14. Chandy T, Sharma CP. Biodegradable chitosan matrix for the controlled release of steroids. *Biomater Artif Cells Immobilization Biotechnol*. 1991;19:745-760.
15. Lesser GJ, Grossman SA, Eller S, et al. The distribution of systemically administered [³H]-paclitaxel in rats: a quantitative autoradiographic study. *Cancer Chemother Pharmacol*. 1995;37:173-178.
16. Brem H, Gabikian P. Biodegradable polymer implants to treat brain tumors. *J Control Release*. 2001;74:63-67.
17. Ringel I, Horwitz SB. Taxol is converted to 7-epitaxol, a biologically active isomer, in cell culture medium. *J Pharmacol Exp Ther*. 1987;242:692-698.
18. Horisawa E, Hirota T, Kawajoe S, et al. Prolonged anti-inflammatory action of D,L-lactide/glycolide copolymer nanospheres containing betamethasone sodium phosphate for intra-articular delivery system in antigen-induced arthritic rabbit. *Pharm Res*. 2002;19:403-410.
19. Liggins RT, Hunter WL, Burt HM. Solid-state characterization of paclitaxel. *J Pharm Sci*. 1997;86:1458-1463.
20. Kemp W. Infrared spectroscopy. In: *Organic Spectroscopy*. London, UK: Macmillan Press; 1996:19-100.
21. Ratner BD, Kwok C. Characterization of delivery systems, surface analysis and controlled release systems. In: Mathiowitz E, eds. *Encyclopedia of Controlled Drug Delivery*. New York, NY: John Wiley and Sons; 1999:261-269.
22. Chenite A, Chaput C, Wang D, et al. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*. 2000;21:2155-2161.
23. Grit M, Crommelin DJ. Chemical stability of liposomes: implications for their physical stability. *Chem Phys Lipids*. 1993;64:3-18.
24. Zuidam NJ, Crommelin DJ. Chemical hydrolysis of phospholipids. *J Pharm Sci*. 1995;84:1113-1119.
25. Kofuji K, Shibata K, Murata Y, et al. Preparation and drug retention of biodegradable chitosan gel beads. *Chem Pharm Bull (Tokyo)*. 1999;47:1494-1496.
26. Schwendeman SP, Costantino HR, Gupta RK, et al. Strategies for stabilising tetanus toxoid towards the development of a single-dose tetanus vaccine. *Dev Biol Stand*. 1996;87:293-306.
27. Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. *Biomaterials*. 1997;18:567-575.
28. Martin A. Polymer science. In: *Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences*. Baltimore, MD: Waverly International; 1994:556-594.
29. Ford JL, Timmins P. *Pharmaceutical Thermal Analysis: Techniques and Applications*. Chichester, UK: Ellis Horwood; 1989:180-200.
30. Nunthanid J, Puttipipatkachorn S, Yamamoto K, et al. Physical properties and molecular behavior of chitosan films. *Drug Dev Ind Pharm*. 2001;27:143-157.
31. Puttipipatkachorn S, Nunthanid J, Yamamoto K, et al. Drug physical state and drug-polymer interaction on drug release from chitosan matrix films. *J Control Release*. 2001;75:143-153.