Chitosan-*Comb-graft*-Polyethylene Glycol Monomethacrylate—Synthesis, Characterization, and Evaluation as a Biomaterial for Hemodialysis Applications

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Received 6 September 2008; accepted 23 May 2009
DOI 10.1002/app.30862
Published online 16 July 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Chitosan was reacted with "Polyethylene glycol monomethacrylate" (PEGm) using a redox initiation method. Different compositions were prepared by varying the relative amount of PEGm in the feed. A maximum of 88% yield with 320% grafting could be achieved. The graft copolymerization was confirmed by FTIR, thermal, and XRD studies. Higher graft % could be achieved as the monomer used is a macro monomer of PEG and the resultant graft is a comb-like polymer. Grafting with PEGm did not affect the thermal stability of chitosan film significantly, however, it resulted in a marginal increase in the tensile strength of the films in the dry state. The products showed much improved swelling at pH 7.4 and pH 1.98 compared to the virgin chitosan. The preliminary biocompatibility evaluation showed that the materials are blood compatible and non-cytotoxic. Though the permeability to low molecular weight solutes like creatinine and glucose was equal to or better than commercial cellulose membranes, the copolymer films expressed comparatively less permeability to these solutes initially, due to the crystalline domains of PEO grafts that impede the transport. On exposure in the medium, this effect is nullified culminating in better permeability. The crystallization of PEG grafts was very helpful in preventing the permeation of the high molecular weight solute albumin, the leakage of which above a certain limit is dangerous to the patient. J Appl Polym Sci 114: 2873–2886, 2009

Key words: grafting; hydrophilicity; glass transition; hemolysis; cytotoxicity

INTRODUCTION

Chitosan is the *N*-deacetylated derivative of chitin, a white hard inelastic nitrogeneous polysachharide found in the outer skeleton of insects, crabs, shrimps, and lobsters and in the internal structure of other invertebrates. Although many polysaccharides possess potentially useful biological activities, relatively few also exhibit material properties that would allow their use as structural materials or tissue scaffolds. Chitosan is one glycopolymer that exhibits both structural potential and useful biological activity. Simple architectural modifications can be used to tune the physical or biological properties of the polymer by creating new intra- or inter-molecular interactions.[1] Chitosan molecule contains a primary amino group at C2 and hydroxyl group at C6 positions, and hence is amenable for a host of chemical reactions under mild conditions (Scheme 1)

Chitosan membranes have been proposed as artificial kidney membranes possessing high mechanical strength in addition to permeability to urea and creatinine. Singh and Ray have extensively reviewed the application of chitosan and modified-chitosans as artificial kidney membranes.[2] The permeability of modified chitosan membranes have been reported by many authors.[3,4] Despite all these potential benefits of chitosan, it has some inherent draw backs. Typically, it is thrombogenic *in vivo* but the modified-chitosans are non-thrombogenic.[5]

Various modifications are suggested to dramatically improve the blood compatibility of chitosan membranes without altering its superior permeability.[6] Chemical modification may become a breakthrough to promote utilization of chitosan and among them; graft copolymerization is anticipated to be a promising approach allowing a wide variety of molecular design.[7,8] Quiet a few articles have been published on the grafting of vinyl monomers on chitosan.[9,10] Recently, we reported the synthesis of chitosan-*g*-poly(HEMA), chitosan-*g*-PMMA, and chitosan-*g*-poly(vinyl acetate) polymers and their properties in biological environment.[11–13] The properties of chitosan can be properly tuned by the nature and concentration of the graft.

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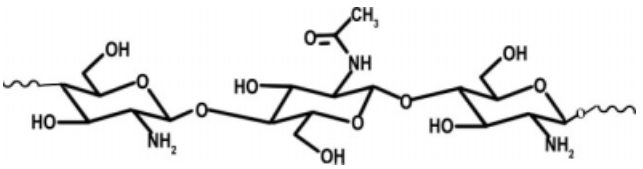


Scheme 1 Structure of chitosan.

Present work describes the synthesis, characetrization, and preliminary biocompatibility evaluation of chitosan-*g*-poly(ethylene glycol monomethacrylate). The membranes are explored for their permeability to low molecular weight solutes like urea, creatinine, and glucose and high molecular weight solute albumin so that they can be proposed as possible hemodialysis membranes.

EXPERIMENTAL

Materials and methods

Chitosan with a deacetylation above 80% and viscosity (of 1% aqueous acetic acid solution at room temperature) above 75 cps was received from India Sea Foods, Cochin (India). Poly(ethylene glycol monomethacrylate) molecular weight 400 was from Polysciences Inc. (Warrington, PA), ceric ammonium nitrate, acetic acid, methanol, sodium hydroxide, etc., were of analytical grade (SD Fine Chemicals Ltd., India). Dulbecco's Minimum Essential Medium (DMEM) was obtained from Sigma Chemical (St. Louis, MO). The Albumin, Creatinine (extra pure) Glucose, and Urea were purchased from SRL Pvt Ltd., India and were used as such.

Grafting

Chitosan was dissolved in 2% aqueous acetic acid by stirring for 48 h at room temperature to make a 2% solution. Hundred milliliter of this solution was placed in a three-necked round bottomed flask, fitted with a condenser and stirrer. Ceric ammonium nitrate (CAN; 0.1*M*) in 10 mL of 1*N* nitric acid was used as the initiator. Grafting was controlled by varying the amount of the polyethylene glycol monomethacrylate (PEGm) and reaction time. The reaction was carried out at 60°C for 4 h under nitrogen atmosphere with constant stirring. Then, the product was precipitated by adding 30 mL of 10% aqueous sodium hydroxide solution with vigorous stirring. The precipitate was washed with distilled water several times, filtered, and dried. The unreacted PEGm was removed from the graft product by soxhlet extraction using methanol until a constant weight was obtained for the product.

The different compositions were coded as CH-P3, CH-P5, and CH-P7.5, the number denoting the weight of the macro monomer (3 g, 5 g, and 7.5 g) in the feed. The % yield and % grafting were calculated using the eqs. (1) and (2).

$$\text{Percentage yield} = \frac{\text{Weight of the graft copolymer}}{(\text{Weight of chitosan} + \text{Weight of PEGm})} \times 100 \quad (1)$$

$$\text{Percentage grafting} = \frac{(\text{Weight of the graft copolymer} - \text{Weight of chitosan})}{\text{Weight of chitosan}} \times 100 \quad (2)$$

Preparation of copolymer films

Chitosan-*g*-PEGm (2 g) was dissolved in 2% aqueous acetic acid with continuous stirring for 24 h. The solution was then poured into disposable polystyrene molds 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 residual sodium hydroxide and dried.

Fourier transform infrared spectral analysis

The FTIR–ATR spectra were used to prove the grafting between PEGm and chitosan. For this purpose, the ATR spectra of the homopolymer of PEGm, chitosan, and chitosan-*g*-PEGm films were taken in the range of 600–4000 cm^{-1} using a Diamond ATR accessory in Nicolet 5700 FTIR Spectrometer.

Thermal studies

Glass transition temperature (T_g) of the chitosan and chitosan-*g*-PEGm films was evaluated by differential scanning calorimetry (DSC 2920 Differential scanning calorimeter, TA Instruments). Thermal stability of the copolymer films was studied on a SDT 2960, (Simultaneous TGA-DTA, TA Instruments). For DSC analysis, 5–10 mg of the samples were crimped inside aluminium sample pans and heated under nitrogen atmosphere at a rate of 10°C/min from −50 to 150°C. The second heat cycle is used for the calculation of glass transition temperature. For

thermogravimetric analysis (TGA), 10–12 mg of the film samples were taken in a platinum cup and heated under nitrogen atmosphere at a rate of 10°C/min from room temperature to 600°C.

Mechanical properties

Tensile properties of the copolymer films were studied using Universal Testing Machine (Instron 1193). The films were conditioned in the testing atmosphere for 48 h. Rectangular strips of 10-mm width were cut and thickness measured using a micrometer. Full-scale load range of 1 KN was applied at a cross head speed of 10 mm/min. Stress at break and percentage elongation were calculated. The computed values are the mean of six repeat measurements.

Swelling properties

Square film samples of 100 mm^2 size (of known mass) were immersed in phosphate buffer at pH 7.4 and aqueous acetic acid at pH 1.98 for known intervals of time. The pieces were removed carefully, blotted between filter paper to remove excess fluid and weighed.

$$\text{Swelling Index} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad (3)$$

Contact angle study

Captive octane in water contact angle of the chitosan and the chitosan-*g*-PEGm films were determined using a contact angle goniometer (GII, Kernco Instruments) to study the hydrophilicity of the films. The films were incubated by putting in doubly distilled water for

24 h after cleaning thoroughly with distilled water. The films were then placed on glass slides and fastened on both ends using teflon tapes. The slide was then immersed in a perspex tank containing double distilled water. The octane droplets were introduced on the surface of the films in water using a micro syringe having a bent needle. The octane/water-contact angles were then measured.

X-ray diffraction patterns

X-ray diffraction patterns of the chitosan and the graft copolymers in the powder form were performed by a wide angle X-ray scattering using Siemens D5005 X-Ray Diffractometer.

Surface morphology of the films

The surface morphology of the chitosan-*g*-PEGm films were studied using an environmental scanning electron microscope, ESEM (Model FEI Quanta 200, Netherlands) and a scanning electron microscope, SEM (Hitachi, Model S 2400). The films were sputter-coated with gold before examining under the SEM.

Blood compatibility of chitosan-*g*-PEGm

In this study, the effects of whole blood contact on WBC, RBC, Platelets, and percentage hemolysis were studied. Blood from human volunteer was collected into anticoagulant, sodium citrate in the ratio 9 : 1. The test materials were fixed in separate wells of siliconised polystyrene petri plate using a small drop of silicone adhesive and allowed it to cure for 24 h. Empty wells were used as the reference. Test materials were immersed in phosphate buffered saline (PBS) before they were exposed to blood. To each well, 2 mL blood was added after aspirating the PBS and a well without any material for reference; 0.5 mL blood sample was immediately withdrawn for initial counts of WBC, RBC, and platelets and also for total hemoglobin, and percentage hemolysis analyzes. The materials were left in blood for 30 min under agitation at 75 ± 5 rpm using an Environ shaker thermo stated at 37 ± 2°C. After 30 min, 1 mL sample was again withdrawn for all the above studies.

The blood samples were centrifuged at 4000 rpm for 15 min and platelet poor plasma was aspirated; 100 μL of the plasma was added to 1 mL, 0.01% aqueous sodium carbonate solution and the absorbances at 380 nm, 415 nm, and 450 nm was measured on a spectrophotometer (Hewlett Packard). Plasma (free) hemoglobin of blood samples were calculated using the eq. (4) below.[14] The total hemoglobin of the blood samples were determined using automatic hematology analyzer. Percentage hemolysis was estimated using the eq. (5).

$$\text{Hemoglobin, mg/dl} = 2 \times A_{415} - (A_{380} + A_{450}) \times 70.26 \quad (4)$$

A_{415}, A_{380}, and A_{450} are the absorbance at 415 nm, 380 nm, and 450 nm, respectively.

$$\%\ \text{Hemolysis} = (\text{Free Hb/Total Hb}) \times 100. \quad (5)$$

The initial cell counts and cell counts after 30 min of exposure of the materials on blood samples were noted using hematology Analyzer Cobas Minos vet (Roche, France) and the count reduction was calculated and compared with that of the reference (Petri plate without material).

The *in vitro* cytotoxicity studies

The *in vitro* cytotoxicity test of the chitosan and chitosan-*g*-PEGm films were carried out by the direct contact assay, MTT assay, and live–dead assay.[15–17]

Direct contact assay

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°C ± 2°C for 24 h ± 1 h, the fibroblast cells with spindle shapes 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.

MTT assay

The quantitative determination of the cytotoxicity of the materials was done by MTT [3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide] assay, which 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. The extract of the films were obtained from 24 h incubation of samples of 100 mm^2 size in tissue culture medium, DMEM, at 37°C. Six thousand 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 films and negative control (high-density polyethylene) 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 (dimethylsulphoxide) 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.

Live–dead assay

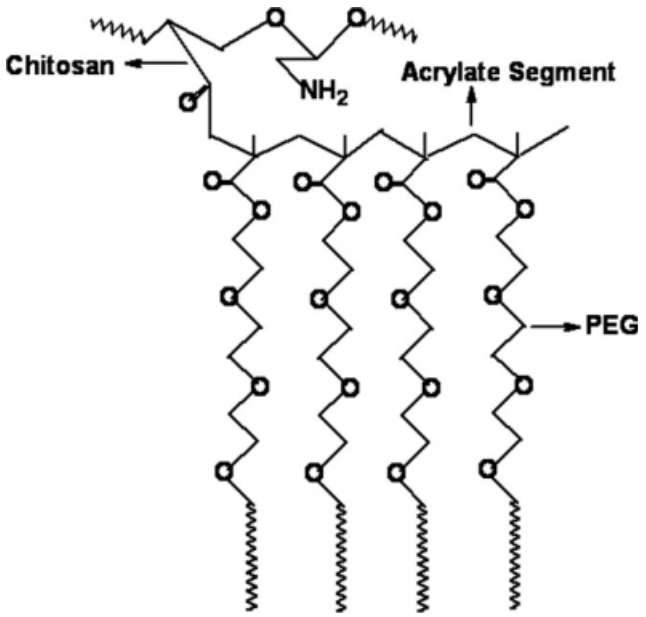
For determining the cell viability, a dual staining technique using acridine orange and ethidium bromide was used. L929 mouse fibroblast cells were seeded onto the materials. After 48 h incubation, the culture medium was removed and the wells were gently washed with PBS; 1 mL of a combination of acridine orange (0.3 μg/mL) and ethidium bromide (0.5 μg/mL) in PBS was added to cover the cells. They were left to incubate for 5 min in the dark at room temperature. The cells were then rinsed gently with PBS and viewed under the fluorescent microscope (Leica DMC 300FX). Viable cells had intact nuclei and stained green/yellow allowing visualization of the nuclear chromatin pattern. Ethidium bromide stains DNA orange, but is excluded by viable cells. Green cells are marked as live and red cells as dead.

Biodegradability of the films

The biodegradation studies of chitosan and the chitosan-*g*-PEGm were carried out in enzyme solutions, trypsin in tris buffer at pH 8.1.[18] Strips of dimensions 8 cm × 0.5 cm of chitosan and the copolymers were immersed each in a separate tube containing 10 mL of 1% enzyme solutions. Films of the same dimensions were put in 10 mL of tris buffer at pH 8.1, as controls. All tubes were kept in an orbital shaker at 37°C. The weights of the strips were taken at definite intervals, e.g., 3 days, 1 week, 2 weeks, and 1 month, after washing with distilled water and subsequent drying at 45–50°C.

Permeation studies

The permeation characteristics of the control and chitosan-*g*-PEGm films for four different solutes creatinine, urea, glucose, and albumin were studied *in vitro* at 37°C. Commercially available cellulose dialysis membrane (cuprophan) was used for comparison. The experimental set up consisted of a two chambered dialysis cell, in which the membrane of known thickness was placed between the two chambers of equal volume. One of the chambers, the donor compartment, was filled with any one of the solutions of known concentration of the solute of interest (creatinine 27.4 mg/L, urea 37 mg/dL, glucose 16.5 m*M*/L, and albumin 8 g/dL) while the other chamber was filled with a solute free buffer



Scheme 2 Comb-like structure of chitosan-*g*-PEGm.

(PBS at pH7.4). The cell was placed on a gentle mechanical shaker which was maintained at 37°C. At appropriate time intervals, 1 mL of the sample was removed from the receptor compartment and replaced with 1 mL of fresh buffer and the amount of the solute diffused through the membrane was measured by standard colorimetric methods namely picric acid method for creatinine, DAM (Diacetyl monoxime) method for urea, *o*-toluidine method for glucose, and Lowry's method for albumin. The amount permeated at a given time t is estimated as C_t, and the permeability coefficient was determined by the eq. (6).

$$P = \ln(2\ C_0/C_t - 1)Vl/2a \quad (6)$$

where P is the permeability coefficient in cm^2/min, C_t is the concentration of the solute in the receptor cell diffused at time t (minute), C_0 is the initial concentration of the solute in the donor cell, V is the chamber volume (cc) of each half cell, l is the thickness of the membrane in cm, and a is the surface area of the membrane in cm^2. The permeability coefficient P is obtained from the slope of the $\ln(2C_0/C_t - 1)\ Vl/2a$ versus time plots.

The amount permeated was calculated using the formula 7.

$$\text{Percentage permeated at time } t = C_t/C_0 \times 100 \quad (7)$$

Where C_t is the concentration of the solute in the receptor cell diffused at time t, C_0 is the initial concentration of the solute in the donor cell, t is the time in minutes.

RESULTS AND DISCUSSION

Grafting

Modification of chitosan through blending or grafting with PEG has been studied by many authors.[19–21] Nasir et al. characterized chitosan-poly (ethylene oxide) blends for hemodialysis membranes.[22] Kolhe and Kannan observed an improvement in ductility of chitosan on modification by blending and grafting with PEG.[23] Composite membranes composed of poly(lactide-*co*-glycolide) and PEG-*g*-chitosan were prepared by electro spinning and were found useful for the sustained release of ibuprofen.[24] Chitosan-poly(ethylene oxide) blend membranes, using different molecular weights of PEO were developed for improved permeability and blood compatibility by Amiji.[4]

In the present study, we used ceric ammonium nitrate as the initiator for the synthesis of chitosan-*g*-PEGm. Maximum yield was obtained after a 4 h reaction. The percentage grafting increased proportionally with the weight of the monomer. A maximum yield of 88% was obtained when the weight of monomer taken was 7.5 gm/2 gm wt of chitosan. The percentage grafting was 320% for the CH-P7.5 composition. As the monomer used is poly ethylene glycol monomethacrylate (macromonomer), the resultant graft has to be comb-like polymer. The schematic of the graft copolymer structure is represented in Scheme 2. The graft copolymer film for the composition CH-P7.5 was found to be very soft and weak and could not be used for membrane type applications. Hence, compositions with higher extent of grafting were not tried. The details of the amount of reactants, reaction conditions, grafting percentage, and yield percentage are given in Table I.

Fourier transform infrared spectral analysis

The FTIR spectra were conducted to investigate the graft copolymerization between chitosan and PEGm. The FTIR spectrum of the homopolymer of PEGm is shown in Figure 1(a). The carbonyl absorption peak appeared at 1727 cm^{-1} for the homopolymer of PEGm. In the graft copolymer chitosan-*g*-PEGm, the presence of the carbonyl absorption peak at

TABLE I
Graft Copolymerization of PEGm Onto Chitosan

S.No.	Polymer reference	Weight of chitosan (g)	Weight of PEGm (g)	Yield %	Grafting %
1	CH-P3	2	3	54.4	36
2	CH-P5	2	5	75.4	164
3	CH-P7.5	2	7.5	88.4	320

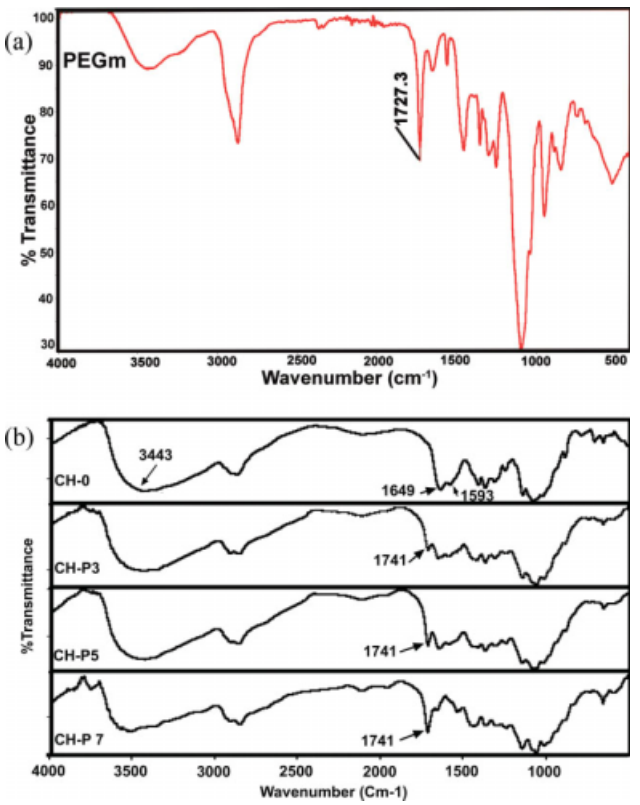


Figure 1 (a) FTIR–ATR Spectra of PEGm homopolymer. (b) FTIR–ATR Spectra of chitosan and chitosan-*g*-PEGm films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1741 cm^{-1} confirmed the grafting reaction between chitosan and PEGm. The shift in the carbonyl absorption peak to higher frequency in the chitosan-*g*-PEGm may be due to the dissociation of the H-bonds in the homopolymer PEGm and the formation of a more ordered comb-like copolymer as shown in Scheme 2. The relative intensity of this peak increased as the grafting % increased in the copolymer composition as we go from CH-P3 (36%) to CH-P7.5 (320%). The characteristic peaks of chitosan like —OH/—NH_2 stretching (3443 cm^{-1}), —C=O— stretching (1649 cm^{-1}), —NH_2 bending (1593 cm^{-1}) are also seen in Figure 1(b).

X-ray diffraction patterns

Wide angle X-ray diffraction patterns of powdered chitosan and the chitosan-*g*-PEGm polymers are shown in Figure 2. Chitosan possesses a certain degree of crystallinity due to its strong inter- and intra-molecular hydrogen bonds among the amino and hydroxyl groups. Chitosan exhibited a typical peak that appeared at $2\theta \sim 20°$. The peak was assigned to the planes (001) and (100).[25] The decrease in the intensity of this peak can be explained by the structural changes due to grafting.

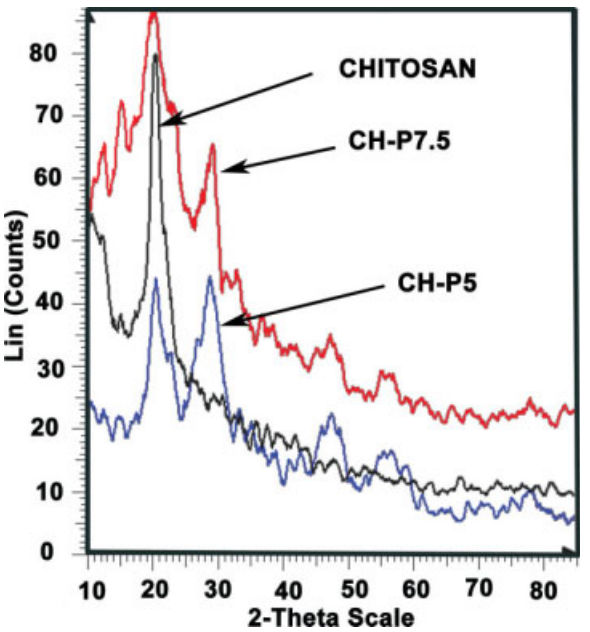


Figure 2 XRD Pattern of chitosan, CH-P5, and CH-P7.5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

When chitosan-*g*-PEGm are formed in the presence of Ce (IV), the amino and hydroxyl groups form a complex to initiate polymerization. This may break the hydrogen bonding between these groups and this result in the decrease in intensity of the crystalline peak in chitosan. However, the graft copolymers manifested additional diffraction pattern at $2\theta = 27°$ and 47° due to the pendant comb-like graft of PEO.

Thermal studies

The DSC analysis of chitosan and chitosan-*g*-PEGm are shown in Figure 3. The DSC scans did not show any distinct glass transition temperature (T_g) for the

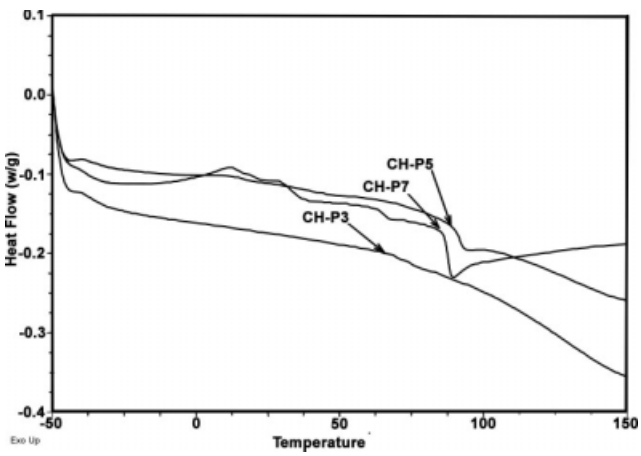


Figure 3 DSC scan of chitosan-*g*-PEGm films (10°C/min in nitrogen atmosphere).

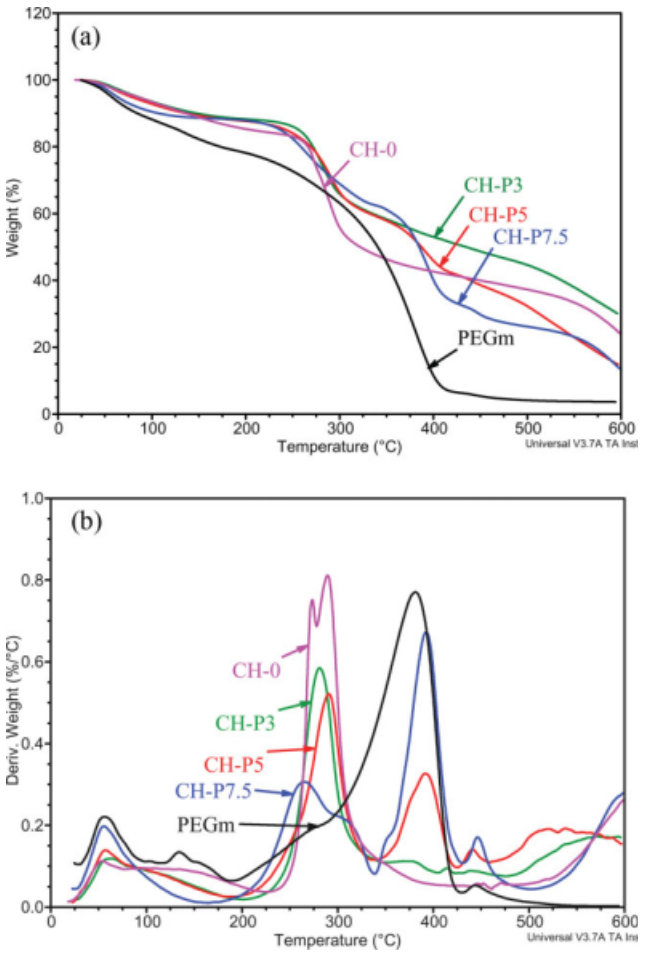


Figure 4 (a) TGA of chitosan-*g*-PEGm films (10°C/min in nitrogen atmosphere). (b) Derivative thermogram of chitosan-*g*-PEGm films (10°C/min in nitrogen atmosphere). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chitosan-*g*-PEGm polymers. On the contrarary, they manifested clear melting endotherm of the PEO segment. As the PEO concentration increased, these endotherms become more prominent. This implies that the chitosan-*g*-PEGm copolymers have a crystalline structure. This is further confirmed from the SEM micrographs and XRD studies.

The thermogravimetric analysis (TGA) of chitosan, PEGm, and the chitosan-*g*-PEGm copolymers of varying compositions are shown in Figure 4(a). It is seen that the copolymers undergo two-stage decomposition corresponding to the chitosan core and the PEGm graft. The derivative thermogram (DTG) of all the copolymer films shown in Figure 4(b) clearly confirm the two-stage decomposition in contrast to the single stage decomposition of virgin chitosan. The second stage decomposition occurring at 390°C is attributed to the PEGm segments as this is comparable to the thermogram of pure PEGm.

TABLE II
The Effect of Grafting on the Decomposition Temperature of Chitosan

Films	T_i (°C)	T_{50} (°C)	T_{end} (°C)	Residue %
CH-0	249	322	599	24
CH-P3	233	435	595	30
CH-P5	224	387	597	15
CH-P7.5	213	385	598	14

The thermal decomposition data are compiled in Table II. Grafting resulted in a decrease in the initial decomposition temperature (T_i) and an increase in the 50% decomposition temperature (T_{50}). As the PEGm grafting percentage increases, the char residue of the graft copolymer decreases at the end of decomposition (T_{end}). This implied a decreased thermal stability of the graft copolymer at elevated temperature. All the analytical data confirm the formation of the graft copolymer.

Mechanical properties

It is seen from Table III that the grafting results in a marginal increase in the tensile strength of chitosan film in the dry state. For CH-P7.5 composition, the film is very soft and weak and the tensile strength is also very low. Percentage elongation is comparatively less for the graft copolymers CH-P3 and CH-P5, in the dry condition (5–9%) and it is increased substantially to 44–45% under wet condition. However, under wet conditions, the copolymers showed a decrease in tensile strength, 6–11 MPa in place of 51 MPa for virgin chitosan. Some studies report that blending or grafting of chitosan with hydrophilic monomer decrease the mechanical property of the chitosan film. Zhang et al. found that when the PEG

TABLE III
Mechanical Properties of the CH-PEGm Films

Sample code	Percentage grafting (wt %)	Tensile strength (MPa)		Percentage elongation	
		Dry	Wet	Dry	Wet
CH-0	0	34.5 ± 11	51.1 ± 7	14.9 ± 7	92.3 ± 6.8
CH-P3	36	46.3 ± 3	11.1 ± 4	4.8 ± 0.8	43.7 ± 3
CH-P5	164	45.4 ± 7	6.5 ± 1	9.3 ± 3	45.4 ± 1
CH-P7.5	320	3.2 ± 1	–	12.8 ± 3	–

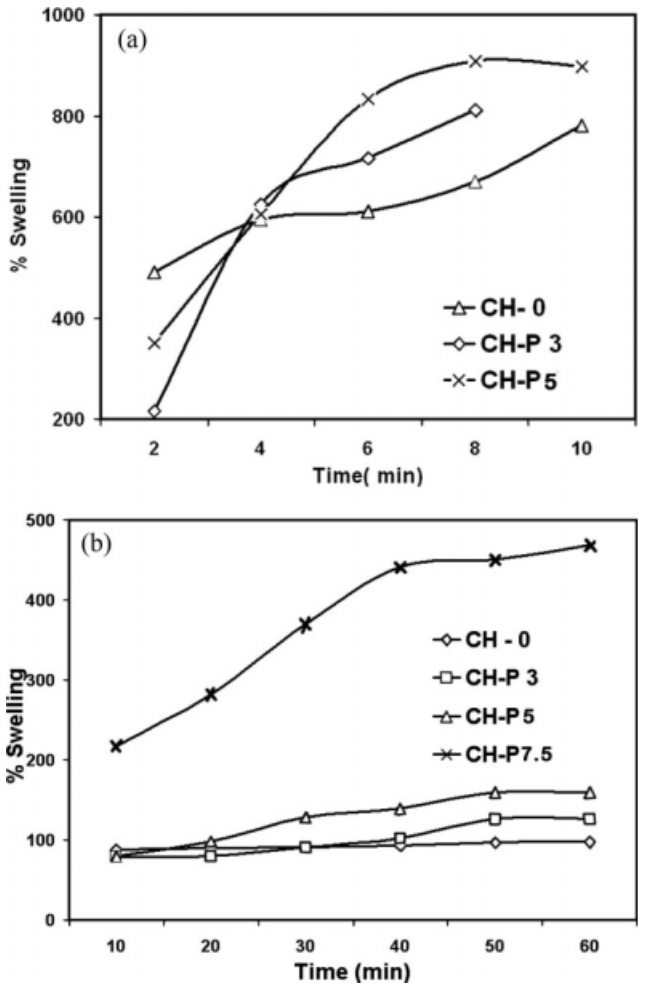


Figure 5 (a) Swelling properties of the chitosan-*g*-PEGm films at pH 1.98. (b) Swelling properties of the chitosan-*g*-PEGm films at pH 7.4.

concentration is increased, the mechanical properties of the chitosan-PEG blends deteriorated.[26] In our studies, it is observed that a moderate grafting helps improve the tensile strength of the copolymer films.

TABLE IV
Effect of Grafting on Octane Contact Angle

Sample	Percentage grafting (%)	Octane-contact angle
CH-0	0	149 ± 1.0
CH-P3	36	157 ± 1.5
CH-P5	164	152 ± 1.5
CH-P7.5	320	Highly hydrophilic

Under wet conditions, the property is found to be significantly decreased. This may be due to the weakening of intermolecular interaction through hydrogen bonding among the chitosan polymer molecules leading to a decrease in the strength of the films. The plasticization effect is reflected in a higher elongation in the wet condition for the graft copolymer.

Swelling studies

The hydrophilicity of the copolymers were assessed by studying their water swelling index and their under water octane-contact angles. The % swelling of the samples were studied in phosphate buffer at pH 7.4 and in aqueous acetic acid at pH 1.98. At pH 7.4, the swelling increased proportional to the extent of grafting as shown in Figure 5(a). A dramatic increase is seen for the graft copolymer CH-P7.5 where the percentage grafting is maximum (320%). In acidic pH, the extent of swelling for the copolymers is substantially high as depicted in Figure 5(b) compared to the value at neutral pH.

Contact angle study

The under water octane-contact angle values are commensurate with the observation from swelling index (Table IV). As can be seen from the table, the hydrophilicity can be improved by grafting PEGm onto chitosan.

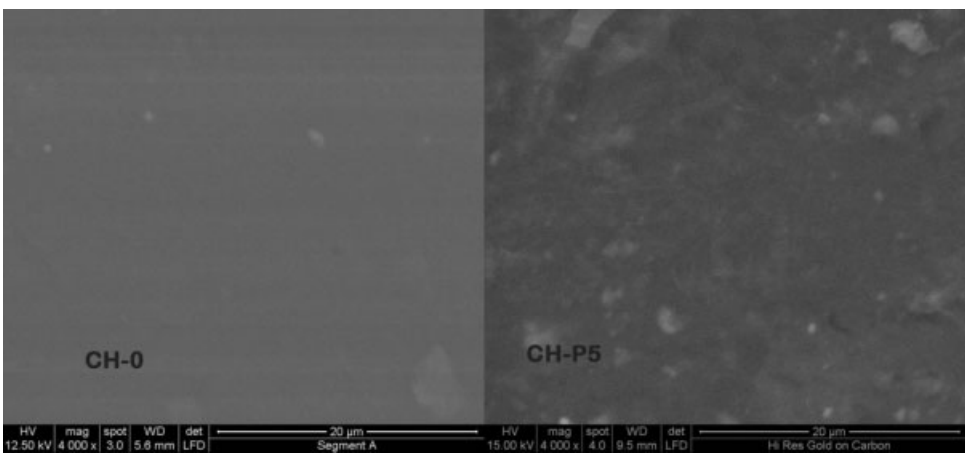


Figure 6 ESEM micrograph of the virgin chitosan (CH-0) and chitosan-*g*-PEGm (CH-P5) films.

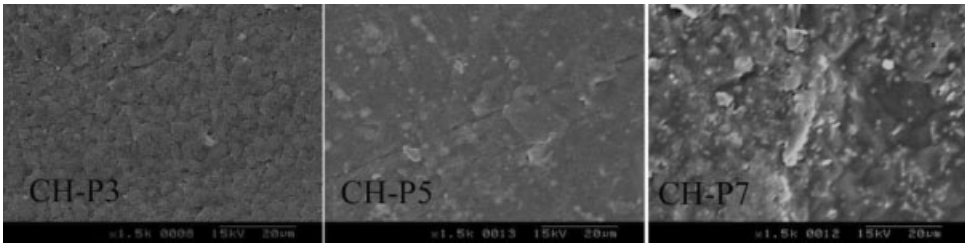


Figure 7 SEM micrograph of the chitosan-*g*-PEGm (CH-P3, CH-P5, and CH-P7.5) films showing crystalline domains.

Surface morphology of the films

The chitosan-*g*-PEGm membranes are non-porous as evident from the ESEM pictures at 4000× magnification (Fig. 6) and the membranes show evidence for crystalline domains in their SEM pictures depicted in Figure 7.

Blood compatibility studies

The cell counts, erythrocytes, leukocytes, and platelets, initial and after 30 min of contact of the materials with the blood are given in Table V. None of the materials caused any significant reduction in the cell counts when the values were compared with that of the reference. The percentage hemolysis of the blood samples on contact with the test materials are given in Table VI. According to ASTM standards, a material with a hemolysis value less than 2% is considered as hemocompatible.[27] Hence, the preliminary blood compatibility studies revealed that the chitosan-*g*-PEGm polymers have potential implications for blood contacting applications.

The *in vitro* cytotoxicity studies

The L929 cells in contact with test samples of chitosan-*g*-PEGm polymer film (e.g., CH-P5) retained their spindle shaped morphology when compared to negative reference material (high-density polyethylene) as shown in Figure 8(a). They did not induce any deleterious effects such as detachment, degenerative and lysis with L929 fibroblasts when placed directly on the monolayer of the cells.

Figure 8(b) depicts that all the copolymer films are highly cytocompatible showing 100% metabolically active cells when compared to the controls (cells without materials). The percentage viability (living cells) values greater than 100% indicate the cell proliferation.

The viability of the fibroblast cells after incubating with the materials for 48 h was assessed by a dual staining technique using a 1 : 1 mixture of acridine orange and ethidium bromide. The live cells stain green and the dead cells deep red when observed under the fluorescent microscope. Figure 8(c) is the fluorescent microscopic image of the stained cells after culturing on chitosan and the copolymer films for 48 h. All the cells on contact with the materials are green confirming that the materials are cytocompatible to L929 mouse fibroblast cells.

Biodegradability of the films

The biodegradation studies of chitosan and the copolymer films were carried out in enzyme solutions, trypsin in tris buffer at pH 8.1 and papain in citrate buffer at pH 6 as detailed in the experimental part. A representative histogram showing the percentage mass-losses for chitosan and chitosan-*g*-PEGm is given in Figure 9. Virgin chitosan films (CH-0) have undergone 28 ± 4.7% mass-loss in the enzyme (trypsin) solution. The chitosan-*g*-PEGm films showed comparatively less mass-loss (5% ± 0.9% to 13% ± 3%) indicating that grafting with PEGm has a control over the biodegradation of chitosan in the enzyme (trypsin) solution. However, it is interesting to note that the chitosan-*g*-PEGm films kept in tris buffer also showed a reduction in their mass (4% ± 0.3% to 8% ± 0.4%) where the chitosan films remain unchanged. This may be due to the increased tendency for hydrolysis of the PEG segment in the aqueous environment. The

TABLE V
Cell Counts in Blood Upon Contact with Chitosan-*g*-PEGm

	RBC count (10^6/mm^3)		WBC count (10^3/mm^3)		Platelet count (10^3/mm^3)	
Sample codes	Initial	After 30 min	Initial	After 30 min	Initial	After 30 min
CH-P3	4.21 ± 0.14	4.26 ± 0.11	6.21 ± 0.14	5.61 ± 0.11	133.5 ± 3.4	125.6 ± 1.7
CH-P5	4.10 ± 0.07	4.25 ± 0.12	6.00 ± 0.14	5.80 ± 0.00	130.3 ± 3.4	120.7 ± 1.9
CH-P7.5	4.17 ± 0.11	4.23 ± 0.15	6.12 ± 0.16	5.65 ± 0.12	132.5 ± 2.9	122 ± 1.5
Reference	4.23 ± 0.25	4.27 ± 0.23	6.27 ± 0.29	5.63 ± 0.31	123.3 ± 0.5.1	116.3 ± 9.3

TABLE VI
Percentage Hemolysis Upon Contact with Chitosan-*g*-PEGm

Sample code	Sample details	Percentage hemolysis
CH-P3	PEGm grafting 36%	0.41 ± 0.19
CH-P5	PEGm grafting 164%	0.39 ± 0.12
CH-P7.5	PEGm grafting 320%	0.42 ± 0.15
Reference	Without sample	0.45 ± 0.13

biodegradability of the films is also assessed from the FTIR spectra of the films after keeping in enzyme solution for a period of 1 month. Figure 10 is the FTIR–ATR spectrum of the chitosan-*g*-PEGm films before and after keeping in enzyme solution (Trypsin) for 1 month. No major difference was visible in the FTIR–ATR spectra of the virgin and treated samples particularly with respect to the NH_2 and CH_2 peaks of chitosan moiety implying the stability of the chitosan moiety towards biodegradation. However, the decrease in intensity of the C=O peak at 1740 cm^{-1} is indicative of the hydrolysis of ester groups of the pendant graft.

Permeation studies

The chitosan-*g*-PEGm polymer films of different compositions, e.g., CH-P3 and CH-P5 films were used for the study. The CH-P7.5 was not explored as the films were too weak to be used for membrane

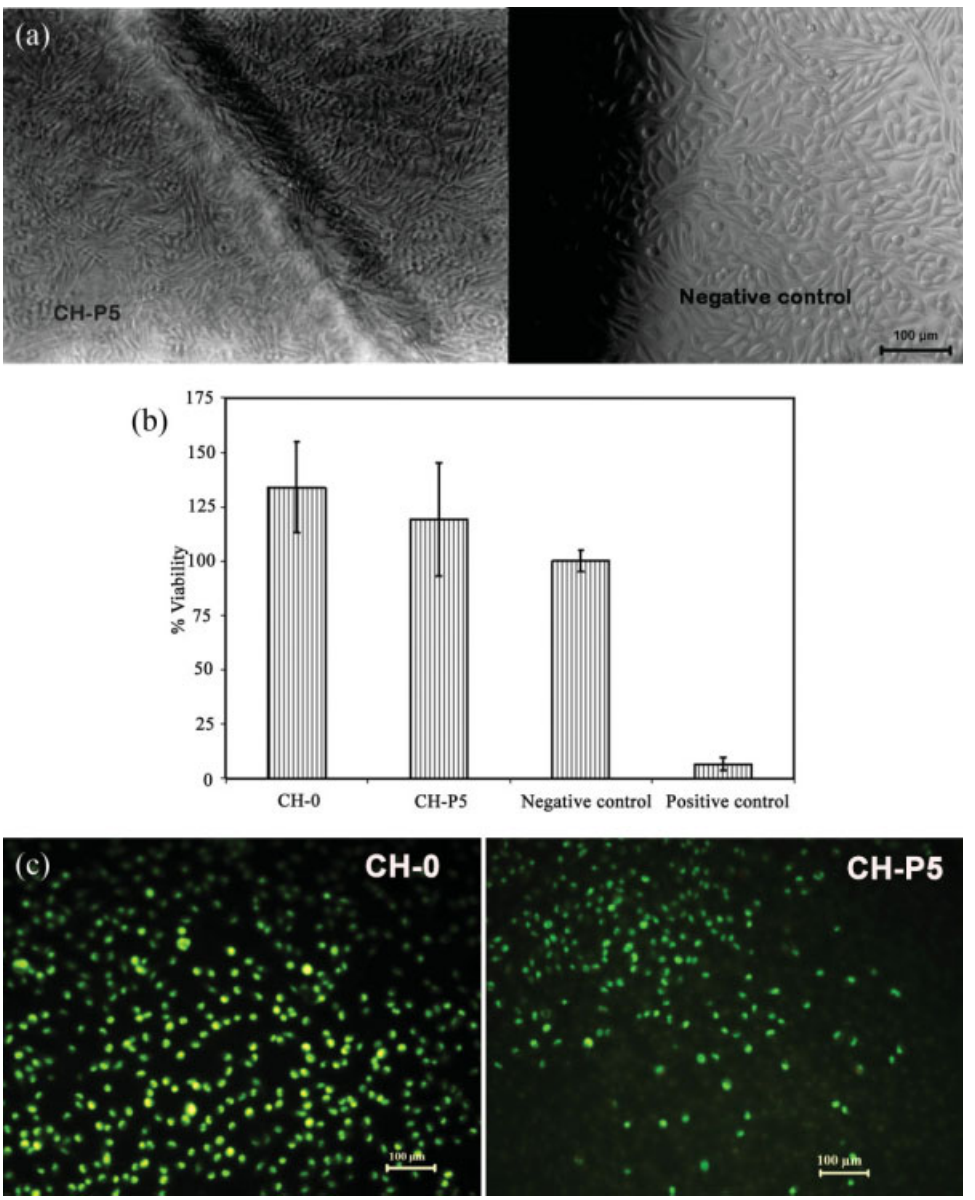


Figure 8 (a) Cytotoxicity evaluation of chitosan-*g*-PEGm film. (b) MTT assay on virgin chitosan and chitosan-*g*-PEGm (CH-P5) films. (c) Live–dead assay on virgin chitosan and chitosan-*g*-PEGm (CH-P5) films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

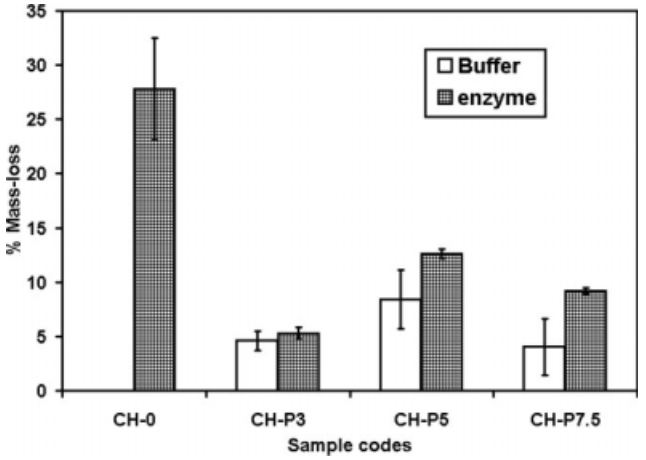


Figure 9 Mass-loss of the chitosan-*g*-PEGm films immersed in trypsin solution and tris buffer for 1 month.

type applications. The permeability coefficient and the percentage of solute permeated are calculated as detailed in the experimental part.

Creatinine permeation

Figure 11 represents a typical curve for the creatinine permeability of chitosan-*g*-PEGm films. Initially, virgin chitosan film shows better creatinine permeability than the chitosan-*g*-PEGm films, CH-P5 and CH-P3. The permeability of molecules across a membrane can be described as a linear function of partition coefficient $P = KD/\Delta x$, where K is the partition coefficient (which is a measure of the solubility of the substance), D is the diffusion coefficient, and Δx is the thickness of the cell membrane. In the case of diffusion through the cell membrane, the substrates that dissolve in lipids (lipid bilayer-cell membrane) pass more easily into the cell. In a similar way the creatinine molecules are soluble in chitosan matrix due to the interaction among the donor

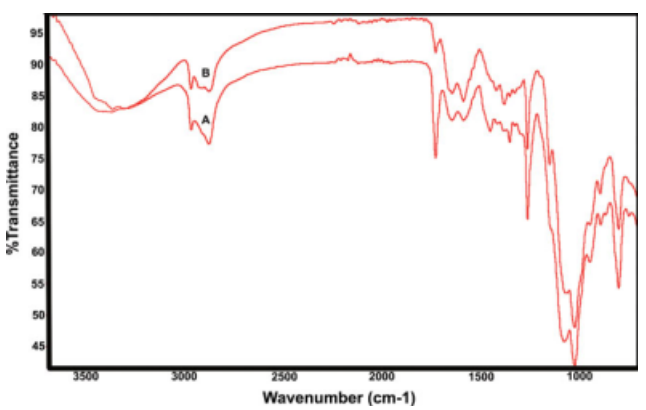


Figure 10 FTIR–ATR Spectra of chitosan-*g*-PEGm(CH-P3) (A) Untreated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

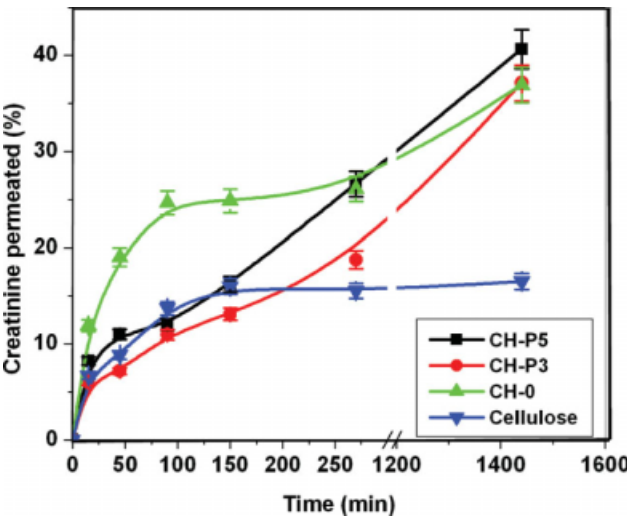
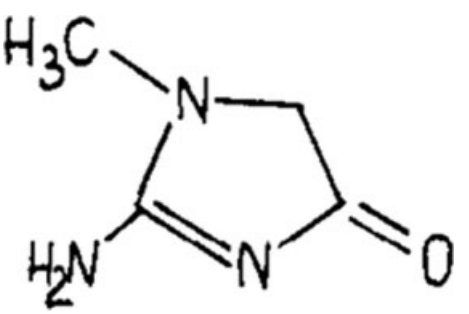


Figure 11 Permeation of creatinine through chitosan-*g*-PEGm films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

groups in creatinine like $-NH_2$, and $-C{=}O$ (Scheme 3) with the free amino groups in chitosan; thereby increasing its concentration in the chitosan matrix leading to its permeability. Similar observations have been reported where the electrostatic attraction between uric acid and chitosan enhanced the permeation of uric acid through the chitosan/PVA blended hydrogel membranes.[28] The CH-P5 composition exhibits a sharp increase after 150 min. The permeability of the CH-P3 composition also increases linearly with time and the value reaches more or less same as that of virgin chitosan film. This is due to the hydrophilic nature of the copolymer films which eventually swell in the aqueous medium leading to the increased permeability of creatinine. It may be noted that both the chitosan-*g*-PEGm and virgin chitosan films exhibit better permeability than the commercial cellulose dialysis membrane.

Glucose permeation

The glucose permeability is high for the medium grafted CH-P3 composition as evident from Figure 12.



Scheme 3 Creatinine.

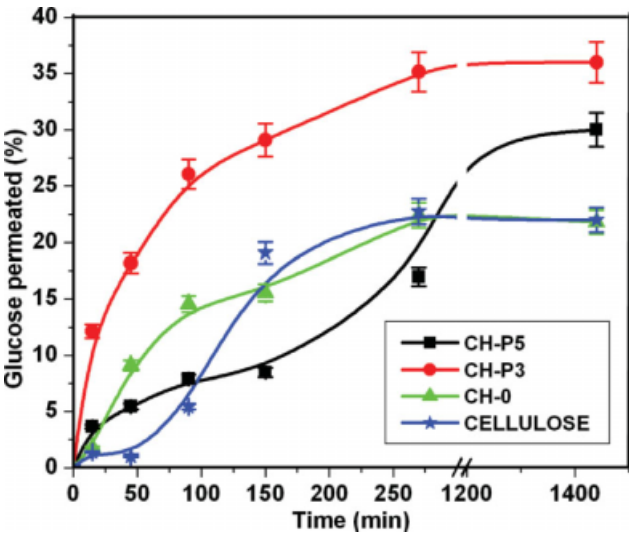


Figure 12 Permeation of glucose through chitosan-*g*-PEGm films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

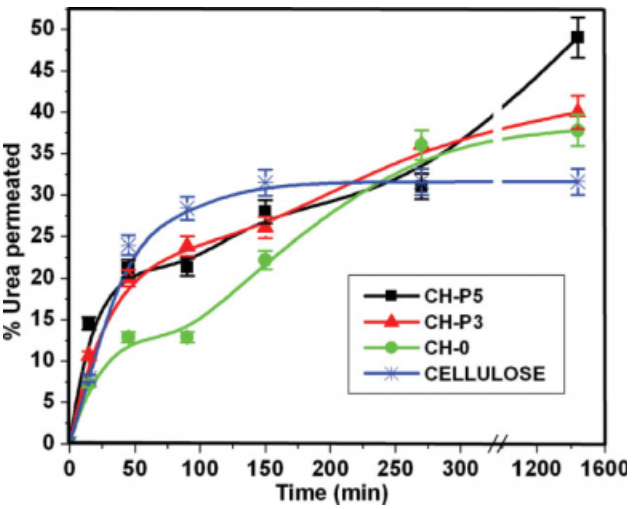
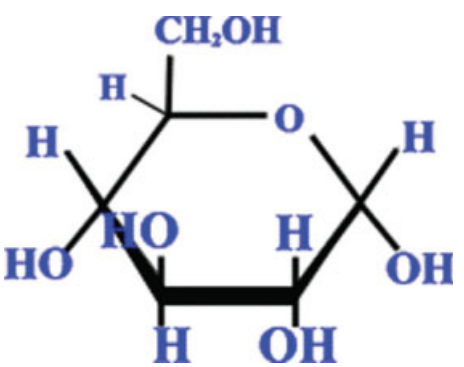


Figure 13 Permeation of urea through chitosan-*g*-PEGm films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Moderate PEO grafting is better for permeation of low molecular weight solutes (Scheme 4). Permeability of glucose is low for the CH-P5 composition proving that higher crystalline content of PEO impedes permeability initially. However, this effect is nullified on prolonged exposure in the diffusion medium. For the CH-P5 composition the amount permeated increased only after 150 min. The glucose permeability of CH-P3 composition increases linearly upto 300 min, after that it reaches a steady state. The maximum amount permeated by CH-P3 and CH-P5 films is 36% and 30%, respectively. It can be seen that virgin chitosan and cellulose films reached the saturation level by 270 min permeating a maximum amount of nearly 23% for both.

Urea permeation

The urea permeability studies through virgin chitosan and chitosan-*g*-PEGm films are depicted in Figure 13. As can be seen from the graph, the chitosan-*g*-PEGm has substantially higher urea permeability than the virgin chitosan film in the initial stages. However, only on prolonged exposure maximum permeability is attained by the copolymer films. Thus, the higher crystalline content of PEO impedes the urea permeation of the chitosan-*g*-PEGm polymers in the initial stages (Scheme 5).

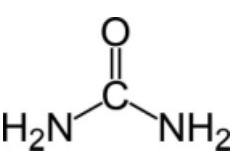


Scheme 4 Glucose. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Albumin permeation

For hemodialysis purposes, the uremic toxins like urea, creatinine, and glucose should be cleared out and the loss of substances essential to the patient should be prevented. Albumin is one of these useful substances, the leakage of which if left unchecked, may compromise the nutritional status of the patient; as a generalization, loss of over about 2–3 g of albumin per treatment session is considered undesirable.[29] None of the chitosan-*g*-PEGm films allowed albumin to pass through them as evident from Figure 14, enabling the films to be used for membrane type applications. Infact, the crystallization of PEG grafts is very helpful in preventing the permeation of albumin. The structure is more effective for albumin as its molecular size is bigger than those of solutes like creatinine, urea, and glucose.

Amiji reported an enhancement of permeability for urea, creatinine, and glucose when studied



Scheme 5 Urea.

through chitosan-poly(ethylene oxide) blend membranes.[4] These blend membranes were having a porous morphology and the increase in permeability was dependent more on the hydrophilicity of the membrane than on porosity. In our study, the chitosan-*g*-PEGm membranes are non-porous and contain crystalline domains as evident from their surface morphology. The crystalline domains impede the permeation of solutes, particularly, the high molecular weight albumin. Similar observations have been reported for the gas permeability of semicrystalline polymers. The crystalline domains provide a tortuous path for the molecules to pass through, thereby enhancing their impermeability.

Table VII shows the permeability coefficient of various solutes through chitosan, chitosan-*g*-PEGm, and the cellulose films. For the medium grafted CH-P3 composition, the permeability coefficient values are higher than the commercial cellulose dialysis membrane.

TABLE VII
Permeation Coefficient of Solutes Through membranes

Films	Creatinine ($P \times 10^5$ cm^2/min)	Urea ($P \times 10^5$ cm^2/min)	Glucose ($P \times 10^5$ cm^2/min)
CH-0	1.9	3.2	4.0
CH-P3	3	4	2.8
CH-P5	1.6	2.3	1.6
Cellulose	2.2	2.1	2.3

CONCLUSIONS

Grafting of PEGm onto chitosan was realized by free radical route. A maximum of 88% yield with 320% grafting could be achieved. The graft copolymerization was confirmed by FTIR, thermal, and XRD studies. Higher graft % could be achieved as the monomer used is a macromonomer of PEG. The grafted films showed enhanced hydrophilicity and overall thermal stability compared to chitosan. The copolymers exhibited improved tensile strength, pH sensitivity, and biocompatibility when compared to virgin chitosan films. The synthesized materials are promising candidates for biomedical applications as hemodialysis membranes, immunoisolatory membranes, and tissue engineering scaffolds.

The chitosan-*g*-PEGm films were explored for their suitability to be used as hemodialysis membranes and found that they had moderately good permeability to low molecular weight solutes like creatinine, glucose, and urea and were impermeable to the high molecular weight solute albumin. The permeability for low molecular weight solutes was comparatively less initially due to the crystalline domain of PEO grafts that impede the transport and on prolonged exposure this effect is nullified culminating in better permeability. However, this was a blessing in disguise for absolutely preventing the transport of high molecular weight solute like albumin.

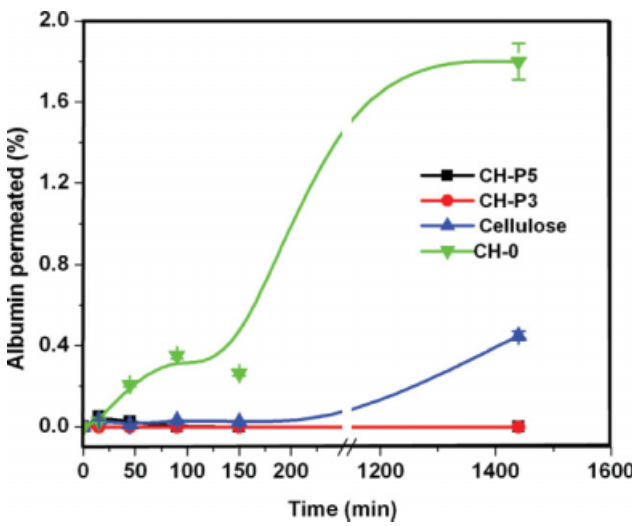


Figure 14 Permeation of albumin through chitosan-*g*-PEGm films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The authors are grateful to the Director, SCTIMST and the Head, B.MT.Wing for providing the facilities, and to "Kerala State Council for Science, Technology & Environment," for the funding.

References

1. Matthew, H. W. T. In Biomimetic Materials and Design; Dillow, A. K., Lowman, A. M., Eds.; Marcel Dekker, INC.: New York, 2006.
2. Singh, D. K.; Ray, A. R.; JMS-Rev Macromol Chem Phys 2000, 40, 1.
3. Li, Y.; Fang, F. Polym Int 2003, 52, 2.
4. Amiji, M. M. Biomaterials 1995, 8, 93.
5. Paneva, D.; Manolova, N.; Rashkov, I.; Danchev, D. J Bioact Compat Polym 2005, 20, 2.
6. Dutta, R. JMS-Polym Rev 2002, 42, 3.
7. Enescu, D.; Olteanu, C. E. Chem Eng Commun 2008, 195, 1269.
8. Zohuriaan-Mehr, M. J. Iranian Polym J 2005, 4, 3.
9. Haung, M.; Fang, Y. Biopolymers 2006, 81, 160.
10. Don, T. M.; King, C. F.; Chiu, W. Y.; Peng, C. A. Carbohydr Polym 2006, 63, 33.
11. Radha Kumary, C.; Divya, G.; Prabha, D. N.; Mathew, S.; Reghunadhan Nair, C. P. J Macromol Sci 2003, 40, 7.
12. Radha Kumary, C.; Prabha, D. N.; Mathew, S.; Reghunadhan Nair, C. P. Trends Biomater Art Org 2005, 8, 2.
13. Radha Kumary, C.; Prabha, D. N.; Mathew, S.; Reghunadhan Nair, C. P. J Appl Polym Sci 2007, 104, 1852.
14. Malinauskas, R. A. Artif Organs 1997, 21, 12.
15. ISO 10993-5: Biological Evaluation of Medical Devices Part 5: Tests for In Vitro Cytotoxicity, 1999.
16. Ciapetti, G.; Cenni, E.; Pratelli, L.; Pizzoferrato, A. Biomaterials 1993, 14, 359.

17. Vento, R.; Giuliano, M.; Lauricella, M.; Carabillo, M.; Di Liberto, D.; Tesoriere, G. Mol Cell Biochem 1998, 185, 7.
18. Smith, R.; Oliver, C.; Williams, D. F. J Biomed Mater Res 1987, 21, 991.
19. Huang, M.; Liu, L.; Zhang, G.; Yuan, G.; Fang, Y. Int J Biol Macromol 2006, 38, 3.
20. Liu, L.; Li, F.; Fanf, Y.; Guo, S. Macromol BioSci 2006, 6, 10.
21. Ganji, F.; Abdekhodaie, M. J. Carbohydr Polym 2008, 74, 3.
22. Nasir, N. F. M.; Zain, N. M.; Radha, M. G.; Kadri, N. A. Am J Appl Sci 2005, 2, 12.
23. Kolhe, P.; Kannan, R. M. Biomacromolecules 2003, 4, 173.
24. Jiang, H.; Dufeifang, B.; Chu, B.; Chen, W. J Biomater Sci Polym Ed 2004, 15, 3.
25. Kim, C. H.; Choit, K. S. J Ind Eng Chem 1998, 4, 1.
26. Zhang, M.; Li, X. H.; Gong, Y. D.; Zhao, N. M.; Zhang, X. F. Biomaterials 2002, 23, 2641.
27. ASTM F 756-00, Annual Book of ASTM Standardsm; ASTM International: West Conhohocken, Pennsylvania, 2000, 309.
28. Yang, J. M.; Su, W. Y.; Leu, T. L.; Yang, M. C. J Memb Sci 2004, 236, 1.
29. Claudio, R.; Bernd, B.; Sudhir, K. B. Hemodialysis Int 2006, 10, S48.