Chitosan-Graft-Poly(vinyl acetate) for Hemodialysis Applications

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Received 6 January 2011; accepted 23 September 2011
DOI 10.1002/app.36261
Published online 19 January 2012 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Chitosan was graft copolymerized with vinyl acetate using ceric ammonium nitrate as the initiator. The chitosan-*g*-poly(vinyl acetate) (chitosan-*g*-PVAc) membranes were found to be blood compatible, noncytotoxic, and biodegradable. The physicochemical characterization of the membranes revealed that the membranes possess the synergistic effect of the natural-synthetic hybrids of chitosan and PVAc with excellent mechanical stability and tunable hydrophilic/hydrophobic characteristics. The permeation characteristics of chitosan-*g*-PVAc membranes for four different solutes creatinine, urea, glucose, and albumin was studied *in vitro* at 37°C for assessment of the suitability of them as hemodialysis membranes. The studies showed that the membranes exhibit higher permeability to creatinine, urea, and glucose compared with the commercial cellulose membrane and are impermeable to the essential nutrient albumin. Hence, the need for the development of biocompatible, mechanically strong dialysis membranes could be addressed with the modification of chitosan through grafting with PVAc. Potential applications like artificial kidney, artificial pancreas, and so forth, are envisaged from these membranes. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 2022–2033, 2012

Key words: graft copolymers; biomaterials; biodegradable; hemocompatible; hemodialysis

INTRODUCTION

Hemodialysis is a method for removing waste products such as creatinine and urea as well as free water from the blood when the kidneys are in renal failure. The mechanical device used to clean the patients' blood is called a dialyzer or an artificial kidney. Although a great deal of research is underway, currently, no viable bioengineered kidneys exist.[1] Regenerated cellulose or cuprophan is widely used as artificial dialysis membrane due to its good solute permeability and mechanical strength. However, the cellulose membranes have little selectivity in the separation of two closely related molecules. The synthetic membranes are good in this respect, but their hemocompatibility is insufficient. Therefore, novel membranes need to be developed for better control of transport, ease of formability, and inherent blood compatibility.[2]

Chitosan is easily derived from chitin, the most abundant natural amino polysaccharide found in the outer skeleton of arthropodes and in the internal structure of invertebrates. The excellent physicochemical and biological properties of chitosan make it suitable for various biomedical applications, like drug carriers, wound dressings, and scaffolds for tissue engineering.[3] Chitosan membranes have been proposed as artificial kidney membranes because of their suitable permeability and high tensile strength.[4] Singh and Ray[5] have extensively reviewed the application of chitosan and modified chitosans as artificial kidney membranes. The permeability of modified chitosan membranes have been reported by many authors.[6–8] Hemodialysis membranes based on functionalized high density polyethylene was synthesized by Rehima.[9] Diffusional properties of chitosan hydrogel membranes cross linked with glutaraldehyde and coated with bovine serum albumin (BSA) was reported by Krajewska.[10] Chen et al.[11] compared the molecular affinity and permeation properties of different molecular weight chitosan membranes. The diffusion properties of caffeine and dextran within chitosan networks were investigated using a gel–gel by Payet et al.[12]

The chitosan modified with 2-hydroxyethylmethacrylate (HEMA) and polyethylene glycol monomethacrylate (PEGm) for hemodialysis applications were reported already by the same authors.[13,14] This study deals with the biocompatibility and permeation properties of chitosan-*g*-poly(vinyl acetate)

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TABLE I
Graft Copolymerisation of Vinyl Acetate Onto Chitosan With Cerium (IV)

No	Weight of chitosan (g)	Weight of VAc (g)	Weight of CAN (mmol)	Time (h)	Monomer conversion (%)	Yield (%)	Grafting %	Polymer reference
1	2	4	1	5	12.5	42	25	CH-V4
2	2	6	1	5	19.3	40	58	CH-V6
3	2	10	1	5	18.4	32	92	CH-V10

(chitosan-*g*-PVAc) membranes. The synthesis and characterization of these membranes have been reported by the same authors in one of the earlier publications.[15]

MATERIALS AND METHODS

Chitosan (deacetylation >80%, viscosity [of 1% aqueous acetic acid solution at room temperature] >75 cps, and molecular weight 120 KDa) was received from India Sea Foods (Cochin, India). Vinyl acetate, ceric ammonium nitrate (CAN), acetic acid, methanol, sodium hydroxide, etc., were of analytical grade (S.D.Fine Chemicals Ltd., India). Albumin, creatinine (extra pure), glucose, and urea were purchased from SRL (India) and were used as such. Urea kit (diacetyl monoxime [DAM] Method) was purchased from Qualigens Diagnostics, for *in vitro* estimation of urea, Manufactured by Sigma Diagnostics (Baroda, India).

Synthesis of chitosan-*g*-PVAc

chitosan-*g*-PVAc were prepared by the method reported earlier.[15] Briefly 2% w/v chitosan in aqueous acetic acid was reacted with varying amounts of vinyl acetate (VAc) in an inert atmosphere using 0.1 M CAN as the initiator. Grafting was controlled by varying the amount of monomer and reaction time. The products were precipitated with aqueous 2% (w/v) sodium hydroxide solution, filtered, and soxhelet-extracted using methanol until a constant weight was obtained for the product and also until the methanol extract was devoid of free PVAc as confirmed by Fourier transform infrared (FTIR) spectroscopy. The different compositions were coded as CH-V4, CH-V6, and CH-V10, where 4, 6, and 10 being the weight in grams of monomer used for synthesis (Table I).

Membrane preparation

The copolymer was dissolved in 2% aqueous acetic acid with continuous stirring for 2–3 days. The solution was then poured into disposable polystyrene moulds and cured at 45–50°C for 48 h. The formed membrane was neutralized with 2% sodium hydroxide solution. It was then removed from the dish and extensively washed with distilled water to remove residual sodium hydroxide. The thickness of virgin chitosan membrane was found to be approximately 0.3 mm and those of the other copolymer membranes was approximately 0.2 mm.

Physicochemical characterizations

The physicochemical characterizations of the copolymers done using FTIR spectrometry, thermal, video contact angle, and X-ray diffraction (XRD) techniques were detailed in the previous communication by the same authors.[15] However, a brief description of the physicochemical characterizations of the copolymers is summarized in the results and discussion part for a quick review.

Sterilization of the samples

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

Blood compatibility test of chitosan-*g*-PVAc

Triplicate test samples were used for exposure to blood and equal number of empty polystyrene petri plates exposed to blood samples was treated as reference. Here, the effect of materials' contact on whole blood to the cell counts like white blood cells (WBC), red blood cells (RBC), and platelets and the percentage hemolysis induced by the materials were studied. Blood from human volunteer was collected into the anticoagulant, sodium citrate in the ratio 9:1. The test materials were fixed in separate wells of siliconized polystyrene petri plate using a small drop of silicone adhesive and allowed it to cure for 24 h. Empty wells were used as 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. A 0.5-mL blood sample was immediately withdrawn

for initial cell counts, total hemoglobin, and percentage hemolysis estimations. 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. The plasma of 100 μL was added to 1mL, 0.01% aqueous sodium carbonate solution and the absorbance at 380, 415, and 450 nm was measured on a spectrophotometer (Hewlett Packard, USA). Plasma (free) hemoglobin of blood samples were calculated using the eq. (1) below.[16] The total hemoglobin of the blood samples were determined using automatic hematology analyzer. Percentage hemolysis was estimated using the eq. (2).

$$\text{Haemoglobin, mg/dL} = 2 \times A_{415} - (A_{380} + A_{450}) \times 70.26 \quad (1)$$

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

$$\% \text{ Haemolysis} = (\text{Free Hb/Total Hb}) \times 100 \quad (2)$$

The cells were counted using a Hematology Analyzer Cobas Minos vet (Roche, France). The count reduction was calculated from the initial cell counts and the counts after 30 min exposure of the materials to the blood samples.

The *in vitro* cytotoxicity studies

The *in vitro* cytotoxicity test of the chitosan and chitosan-*g*-PVAc films were carried out by the direct contact assay, MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide] assay, and live–dead assay as detailed in the following paragraphs.[17–19]

Direct contact assay

Cleaned and ETO sterilized film samples of surface of area 0.1–0.3 cm^2 were used for the direct contact test. Test samples, negative controls (high density polyethylene), and positive controls (copper) in triplicate were placed on subconfluent monolayer of L-929 mouse fibroblast cells [source American Type Culture Collection (ATCC)] and cell passage 5. After incubation of the cells with test samples at 37 ± 2°C for 24 ± 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 noncytotoxic; 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 assay, which is a standard colorimetric assay for the quantification of living cells. Yellow tetrazolium salt MTT is reduced to purple colored formazan by the enzymes present in the mitochondria of the metabolically active cells. The absorbance of the colored formazan solution is measured at 570 nm.

MTT dissolved at a concentration of 5 mg mL^{-1} in sterile PBS, 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. Dilution of 1:10 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, Dulbecco's modified Eagle medium (DMEM), at 37°C. Approximately 6000 cells in 100 μL tissue culture medium were plated in a 96-well flat-bottom tissue culture plate. The plate was then incubated at 37°C in 5% carbon dioxide overnight. When monolayer was attained, culture medium was removed, rinsed with PBS, and 100 μL each of the extracts of copolymer films, high-density polyethylene (negative control), 100 μL of diluted phenol, and 0.1% (v/v) in tissue culture medium, (positive control) were added to different prelabeled 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 multichannel 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 the 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. One milliliter of a combination of acridine orange (0.3 μg mL^{-1}) and

ethidium bromide (0.5 μg mL^{-1}) 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).

Surface topography of the blend films by ESEM and AFM

The surface morphology of the chitosan-*g*-PVAc films was studied using an environmental scanning electron microscope (ESEM; Model FEI Quanta 200, The Netherlands) and the surface topography was studied using an Agilent 5500 atomic force microscope (AFM) in the contact mode. Microfabricated silicon cantilever tips (PPP-N9832-80002; Agilent technologies) was used for the measurement at a scan rate of 1 Hz.

Permeation studies

The permeation characteristics of the control and chitosan-*g*-PVAc films (thickness approximately 0.2 to 0.3 mm) for four different solutes creatinine, urea, glucose, and albumin were studied *in vitro* at 37°C. Commercially available cellulose hemodialysis membrane was used for comparison. The experimental set up consisted of a two-sided dialysis cell with 95 mm length and 65 mm width and the middle portion of each side has a cylindrical hollow chamber with 42 mm length and semicircular edges of 10 mm radius. The membrane of known thickness was placed between the two chambers of equal volume. One of the chambers, the donor chamber, was filled with solutions of known concentration of the solute of interest (creatinine 27.4 mg L^{-1}, urea 37 mg dL^{-1}, glucose 16.5 mM L^{-1}, and albumin 8 g dL^{-1}), one solution at a time, while the other chamber was filled with a solute free buffer (PBS at pH 7.4). 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 method for urea, *o*-toluidine method for glucose, and Lowry's method for albumin.[20–22]

The amount permeated was calculated using the eq. (3).

$$\text{Percentage permeated at time } t = C_t/C_0 \times 100 \quad (3)$$

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 and t is the time in minutes.

The permeability coefficient of chitosan and the modified chitosan membranes were calculated using the equation

$$P = \ln (2\ C_0/C_t - 1)\ V1/2a \quad (4)$$

where P is the permeability coefficient in $cm^2\ min^{-1}$, 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 (2 C_0/C_t − 1) Vl/2a versus time plots.

Each set of experiment was carried out in triplicate and results were expressed as mean ± standard deviation. Similar results within the maximum error of 2–3% were reported.

RESULTS AND DISCUSSION

Synthesis of chitosan-*g*-PVAc

Chitosan-*g*-PVAc copolymers were synthesized by a free radical technique using CAN as the initiator.[15] A maximum of 40% yield with 92% grafting could be achieved. The details of the amount of reactants, grafting percentage, and yield percentage are given in Table I.

Physicochemical characterizations

FTIR spectral analysis

The FTIR spectra of the chitosan-*g*-PVAc showed the presence of the carbonyl absorption band at 1741 cm^{-1}, which confirmed the presence of PVAc grafts. Amides I and II bands of chitosan are located at 1649 and 1593 cm^{-1}, respectively, and the symmetric stretching vibrations of the NH_2 groups of chitosan at 3443 cm^{-1} as seen in Figure 1.

XRD patterns

Wide angle XRD patterns of powdered chitosan and a typical copolymer CH-V10 were shown in Figure 2. Chitosan exhibited the major crystalline peak at $2\theta = 20°$ in agreement with the literature values.[23] In graft copolymers, additional diffractions are seen at $2\theta = 28°$, 48°, and 58°. Although each of these peaks has not been assigned to any specific planes, the differing pattern of the copolymer *vis-à-vis* that of the parent molecule is indicative of a clear structural change in chitosan as a result of grafting with PVAc.

Thermal studies

Chitosan shows a glass transition temperature (T_g) at 62°C. For the chitosan-*g*-PVAc, when the PVAc

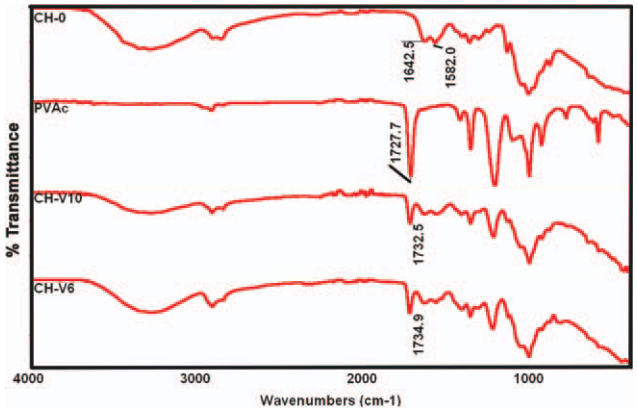

Figure 1 FTIR spectra of Chitosan, PVAc, CH-V10, and CH-V6. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

graft is less as in the case of CH-V4 (25%), no detectable T_g is seen. However, as the PVAc content gets enhanced, a single transition is seen in the copolymers CH-V6 and CH-V10 at 28 and 29°C, respectively (Fig. 3 and Table II).

Figure 4(a) shows the TGA for chitosan and the copolymers. The initial decomposition temperature of both the copolymers are not significantly affected due to grafting. The two-stage mass loss pattern of the copolymers confirms grafting, which is more distinct in the derivative thermogram (DTG) as seen in Figure 4(b).

Mechanical properties

As per the data given in Table III, the grafting results in an enormous increase in the tensile strength of chitosan membrane in the dry state with not much change in percentage elongation. The percentage elongation increased considerably in the case of the graft copolymers under wet condition as

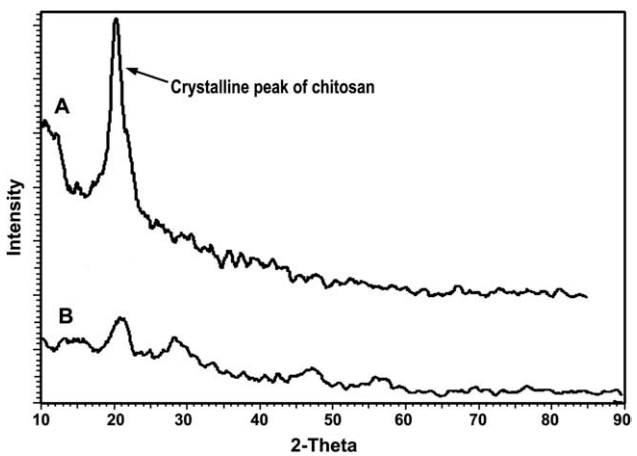

Figure 2 Typical XRD pattern of (A) chitosan and (B) CH-V10.

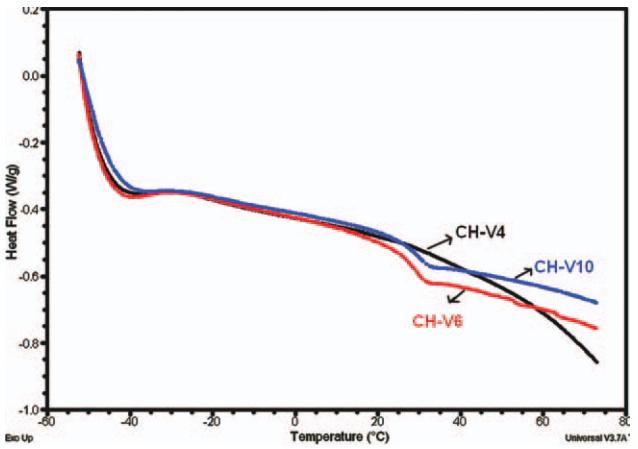

Figure 3 DSC scans of CH-V4, CH-V6, and CH-V10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the VAc segment retards the rigidity of the chitosan chain, making the copolymer more soft and extensible. The maximum achieved tensile strength of the chitosan-*g*-PVAc films were found to be 123 ± 16 MPa in the dry state and 59 ± 10 MPa in the wet state. The poor mechanical properties and the consequent difficulty in processing of chitosan could be obviated by way of graft copolymerization of VAc onto chitosan.

Swelling studies

The hydrophilicity of the copolymers is assessed by studying their water absorption capacity and their under-water octane contact angles. It is already reported that chitosan is hydrophobic in nature and its swelling index at pH 7.4 is very low. On grafting the hydrophobic monomer (i.e., VAc), chitosan is expected to increase hydrophobicity, which is confirmed by our results. Figure 5 shows that all the chitosan-*g*-PVAc copolymer films except CH-V4 composition have less percentage swelling when compared with ungrafted chitosan films. In CH-V4, the percentage grafting is only 25%; it is concluded that moderate PVAc grafting is conducive to enhance the swelling characteristics. Water absorption occurs by way of H bonding of NH_2 and OH groups on chitosan, with water. When the PVAc content is enhanced beyond 25%, possibly the OH

TABLE II
The Effect of Grafting on the T_g of Chitosan

Sample codes	T_g (°C)
PVAc	28
CH-0	62
CH-V4	No specific T_g
CH-V6	28
CH-V10	29

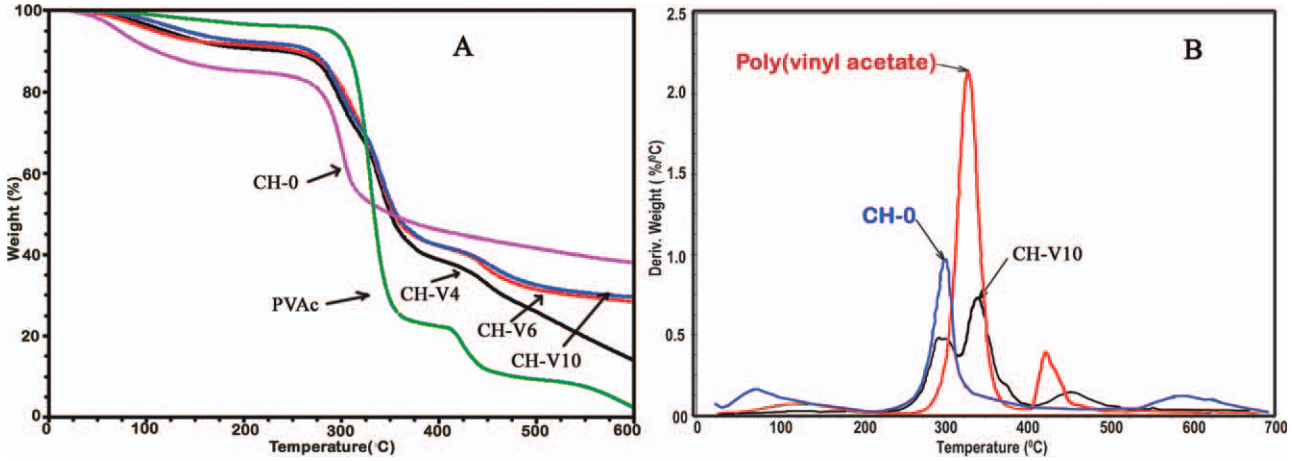

Figure 4 A: TGA of PVAc, CH-0, CH-V4, CH-V6, and CH-V10 and (B) typical DTG of CH-0, PVAc, and CH-V10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and NH_2 groups are engaged in H bonding with acetate groups; thereby decreasing their affinity for H bonding with water.

Octane contact angle. The under-water octane contact angle values shown in Table IV also support the fact that the hydrophobicity of the membranes increase on increasing the extent of grafting.

Blood compatibility test of materials

The RBC, WBC, and platelet counts (initial and after 30 min of contact of the materials with blood) are given in the Table V. From the data, it is clear that when compared with the reference material, none of the test materials caused a reduction in the cell counts as an effect of contact with the test materials. Table VI shows that all the chitosan-*g*-PVAcs have hemolysis values ranging from 0.33 to 0.38%. According to ASTM standards, a material with a hemolysis value <2% is considered as hemocompatible.[24] Thus, the blood compatibility studies of the materials show that the chitosan-*g*-PVAc have potential implications for blood contacting applications. Ching et al.[25] also reported similar observations that chitosan/heparin polyelectrolyte complex immobilization can endow the poly(acrylonitrile) membrane hemocompatibility and antibacterial activity, while retaining the original permeability.

TABLE III
Mechanical Properties of the Chitosan-*g*-PVAc Membranes

	Tensile strength (MPa)		Elongation (%)	
Sample code	Dry	Wet	Dry	Wet
CH-0	35 ± 11	51 ± 7	15 ± 7	92 ± 7
CH-V4	123 ± 16	25 ± 4	11 ± 3	153 ± 7
CH-V 6	96 ± 17	55 ± 7	27 ± 10	151 ± 20
CH-V10	117 ± 0.9	59 ± 10.2	13 ± 0.4	163 ± 8

In vitro cytotoxicity test

Direct contact assay

Figure 6 shows the image of the L929 fibroblast cells when exposed to chitosan-*g*-PVAc films and the morphology of normal fibroblast cells grown on a negative control (high density polyethylene). One of the most sensitive toxicity testing protocol is based on the direct contact of the sample with the cell culture. Normal L929 fibroblast cells are spindle shaped, healthy, and viable with a glistening appearance. Nontoxic materials on contact with such cells would preserve the morphological aspects of the cells to larger extent, while the materials with cytotoxicity would cause the cells to undergo lysis, lose their spindle shape, and become more rounded. The L929 cells in contact with test samples retained their spindle-shaped morphology when compared with negative control. They also did not induce any deleterious effects such as detachment, degenerative and

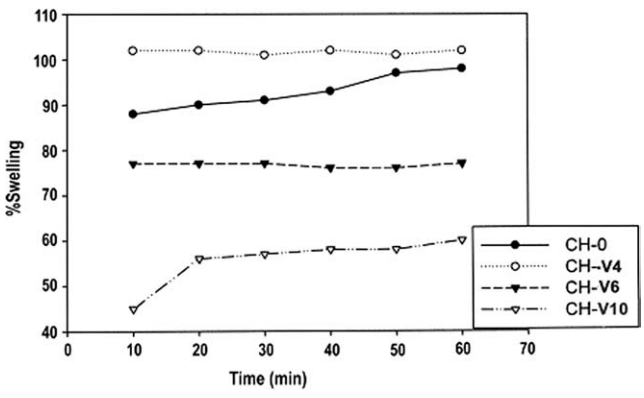

Figure 5 Swelling percentage of chitosan and chitosan-*g*-PVAc at pH 7.4.

TABLE IV
Effect of Grafting on Octane Contact Angle of Chitosan

Sample	Octane contact angle (°)
CH-0	149
CH-V4	145
CH-V6	91
CH-V10	92

lysis with L929 fibroblasts when placed directly on the monolayer of the cells indicating that the materials are cytocompatible.

MTT assay

Figure 7, the MTT assay, depicts that all the copolymers are highly cytocompatible showing 100% metabolically active cells when compared with the controls (cells without materials). The percentage viability (living cells) values greater than 100% indicate the cell proliferation.

Live–dead assay

Figure 8 is the fluorescent microscopic image of the stained cells after culturing on chitosan and the chitosan-*g*-PVAc for 48 h. All the cells on contact with the materials are green confirming that the materials are cytocompatible to L929 mouse fibroblast cells. Viable or live cells have intact nuclei and stain green with acridine orange. Ethidium bromide stains DNA orange, but is excluded by viable cells. Hence, green cells are marked as live and red cells as dead. Thus, the chitosan-*g*-PVAc polymers have proved to be cytocompatible as evident from the direct contact, MTT assay, and live–dead assay.

Surface morphology of the films

The ESEM images at 4000× magnification depicted in Figure 9 show that the chitosan-*g*-PVAc films have a nonporous surface morphology. The morphology of the films was observed at 24°C by AFM as depicted in Figure 10. No sign of pore structure could be observed at a 1μm scale.

Permeation studies

When no chemical reaction between the solute and membrane takes place, solute diffusion through hydrogel membranes is described by Fick's first law, $J_s = P/l\ \Delta c$.[10] Where J_s is the solute flux (g cm^{-2} S^{-2}), P is the solute diffusion permeability coefficient in the membrane (cm^{-2} S^{-1}), l is the membrane thickness, and Δc is the solute concentration difference across the membrane (g cm^{-3}). To interpret the membrane diffusion data, two models of solute diffusional transport, namely, capillary pore model and free volume model are used. In the capillary pore model, fixed capillary pores perpendicular to the membrane surface can be visualized. However, no evidence of pores has been observed for the chitosan-*g*-PVAc membranes by either ESEM or AFM studies. Due to copolymerization with VAc, the T_g of chitosan decreases as evident from Table II and this internal plasticization increases the free volume of the chitosan chains. In the free volume model, the diffusion of solutes occur by successive jumps through the free volume that are larger than the solute molecules. The permeation of each solute through the membranes is detailed in the following sections.

Creatinine permeation

From Figure 11, it is clear that the CH-V4, CH-V6, and CH-V10 copolymer membranes show higher permeability to creatinine than the virgin chitosan and the commercial cellulose membranes. During the first 50 min, 20–35% of the initial amount of creatinine is permeated through the chitosan-*g*-PVAc membranes. In the case of commercial cellulose membrane, equilibrium is attained at 90 min itself, but for the copolymer membranes (CH-V4 and CH-V10), the amount permeated goes on increasing even after 4 h.

In our early studies, a marginal decrease in creatinine permeation was observed for the graft copolymers of chitosan, modified with the hydrophilic vinyl monomers, chitosan-*g*-poly(HEMA), and chitosan-*g*-PEGm compared with virgin chitosan membranes.[13,14] In the case of chitosan-*g*-PVAc, it is

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

	Total RBC count ($\times 10^6$ mm^{-3})		Total WBC count ($\times 10^3$ mm^{-3})		Platelet count ($\times 10^3$ mm^{-3})	
Sample code	Initial	After 30 min	Initial	After 30 min	Initial	After 30 min
CH-0	6.81 ± 0.3	6.12 ± 0.3	6.55 ± 0.3	6.52 ± 0.3	118.60 ± 6.3	115 ± 7.4
CH-V4	4.31 ± 0.03	4.08 ± 0.1	5.83 ± 0.1	5.87 ± 0.3	130.33 ± 3.4	121.67 ± 8.5
CH-V6	5.86 ± 0.10	5.53 ± 0.30	5.66 ± 0.10	5.25 ± 0.40	122.35 ± 11.00	123.57 ± 7.40
CH-V10	5.40 ± 0.5	5.60 ± 0.4	5.40 ± 0.6	5.60 ± 0.4	119.33 ± 15.3	109.33 ± 12.6
Reference	6.27 ± 0.29	5.63 ± 0.31	6.27 ± 0.29	5.63 ± 0.31	123.33 ± 5.13	116.33 ± 9.29

TABLE VI
Percentage Hemolysis Values of Chitosan-*g*-PVAc

S.No.	Sample code	Hemolysis (%)
1	CH-0	0.33 ± 0.11
2	CH-V4	0.33 ± 0.17
3	CH-V6	0.35 ± 0.16
4	CH-V10	0.38 ± 0.18
5	Reference	0.45 ± 0.10

interesting to note that all the copolymer membranes show enhanced permeability to creatinine compared with virgin chitosan membranes. This could be interpreted as follows. The chitosan-*g*-PVAc membranes behave as a rubbery polymer at the physiological temperature as evident from their T_gs (28–29°C). Segmental mobilities are increased in this state effecting in an increased free volume. When a penetrant diffuses into a rubbery polymer above its T_g, a rapid adjustment of the polymer chains occurs and as a result, a fast diffusion results. Fickian behavior is usually exhibited in such cases.[26] The movement of the diffusant is without any interactions and is simply based on a random walk. On the other hand, the glassy polymers respond slowly initially. Eventually, they swell in the diffusion medium and the polymer structure changes as diffusion proceeds.

Urea permeation

In the case of urea permeation also, 20–35% of the initial amount is permeated through the chitosan-*g*-PVAc membranes at 50 min (Fig. 12). Hence, the softening of the chitosan-*g*-PVAc polymers above the T_g and the consequent increase in the free volume has improved their urea permeability. In the case of the glassy polymers chitosan-*g* poly(HEMA) and chitosan-*g*-PEGm, initially all the copolymer films showed a marginal decrease in the urea permeated, *vis-à-vis* cellulose film.[13,14] The amount permeated is increased for these films only after the diffusion process advanced during which time the glassy polymers swelled in the aqueous medium.

Glucose permeation

The glucose permeability of the chitosan-*g*-PVAc membranes is only 6–11% at the first 50 min. This may be due to the higher molecular radius of glucose (5.5 A°) compared with the smaller radii of urea (1.8 A°) and creatinine (3 A°). The CH-V4 copolymer exhibits the maximum glucose permeability as shown in Figure 13. All the other copolymers as well as virgin chitosan show higher rate of permeation initially than the commercial cellulose. After 150 min, they show similar trend and the amount permeated becomes more or less the same.

Thus, we have seen that the permeability of small molecular weight solutes like creatinine, urea, and glucose is higher in the chitosan-*g*-PVAc films than virgin chitosan and commercially available cellulose films. The permeability coefficient of all the membranes calculated as per eq. (4) is given in Table VII. We could not find any significant difference or specific tendency in the permeability of various solutes among the chitosan derived copolymer membranes

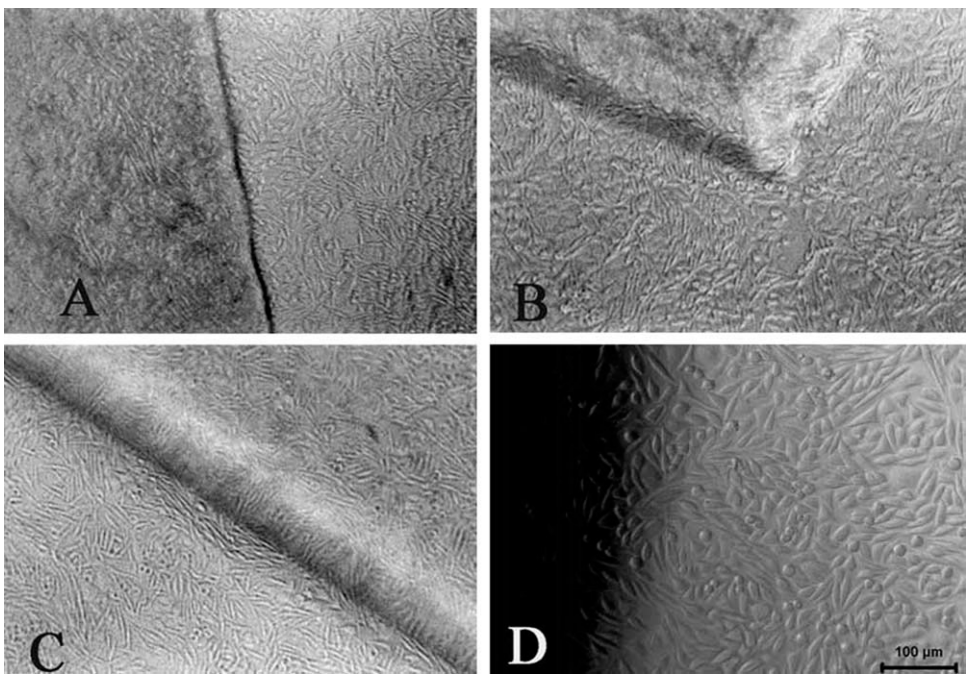

Figure 6 L929 cells in direct contact with (A) CH-V4, (B) CH-V6, (C) CH-V10, (D) negative control for 24 h.

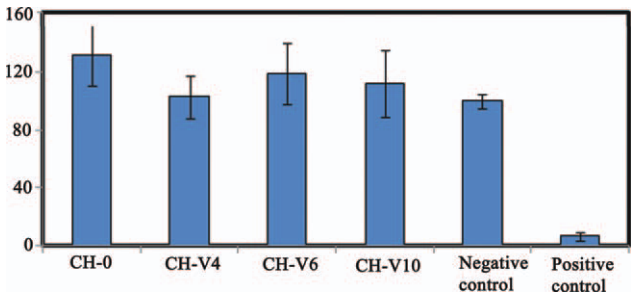

Figure 7 MTT assay on CH-0 and chitosan-*g*-PVAc. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Table VII). This may be due to the fact that the T_g (28–29°C) and the percentage elongation (153 ± 7%) to (163 ± 8%) of the copolymers were invariant with the extent of grafting. Hence, all the copolymer films are having similar elastic nature with more or less similar permeability characteristics at the experimental temperature of 37°C.

Albumin permeation

As depicted in Figure 14, chitosan is permeable to albumin and the amount permeated is nearly 2% of the initial concentration (8 g dL^{-1}). For hemodialysis purposes, the uremic toxins like urea and creatinine should be cleared out and the loss of substances essential to the patient should be prevented. Albumin is one of the 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.[27] The chitosan-*g*-PVAc films allowed only trace amount of albumin to pass through them, for example, CH-V4 (0.0024 g), CH-V6 (0.004 g), and CH-V10 (0.048 g) and the amount is well within the allowed range of albumin leakage.

Several reports are there in the literature relating the polymer properties and semipermeability. One of the studies stated the usefulness of hydrophilic polymers as semipermeable membranes as in the case of a film formed from an aqueous solution of poly(vinyl alcohol) and chitosan salt.[28] In another study, Nasir et al.[29] reported that Poly(ethylene oxide)-chitosan blends have porous structure compared with the pure chitosan membranes, and this increased the permeability of the blend membranes. According to them, the hydrophilicity of the membranes, molecular weight, and chemical nature of the metabolites are the important parameters in determining their transport properties. Amiji studied the permeability properties of chitosan-poly(ETO) blends and reported that the increase in permeability through the blend was dependent more on the hydrophilicity of the membrane than on its porosity.[30] The chitosan-*g*-PVAc films of our study are not porous as evident from the ESEM and AFM images (Figs. 9 and 10), respectively, and they are neither hydrophilic as evident from their underwater octane contact angle values nor swelling index as already reported.[15] Hence, the permeation of low molecular weight solutes through these membranes can not be by pore type mechanism as explained by Singh and Ray[31] in their study of glucose permeation through poly(HEMA) modified chitosan membranes. Hydrophilicity has also no role in improving the permeability of solutes through these membranes. As already explained, it is observed that chemical modification of chitosan through grafting with PVAc has increased the extensibility (elongation) of chitosan membranes from 93% to 150–163% in the wet condition.[15] Additionally, the T_gs of CH-V6 and CH-V10 films are 28 and 29°C, respectively, which is well below the experimental temperature of 37°C. Hence, the copolymer membranes behave as a rubbery matrix with increased segmental mobility at 37°C. This chain relaxation enables the low molecular weight solutes like urea, creatinine, and glucose to pass through the membranes by a mechanism of "random walk" similar to water permeability through lipid bilayer. As albumin is a high molecular weight solute, these membranes are impermeable to it.

From the studies conducted so far, it is clear that the membranes prepared from chitosan-*g*-PVAc are non porous and hydrophobic and their low molecular weight-solute permeability are superior to the commercial cellulose membranes. Moreover, these

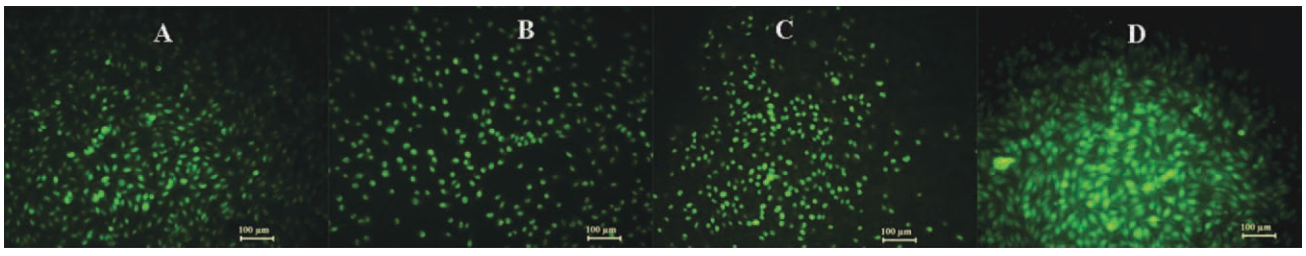

Figure 8 Live–dead assay on (A) CH-0, (B) CH-V4, (C) CH-V6, and (D) CH-V10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

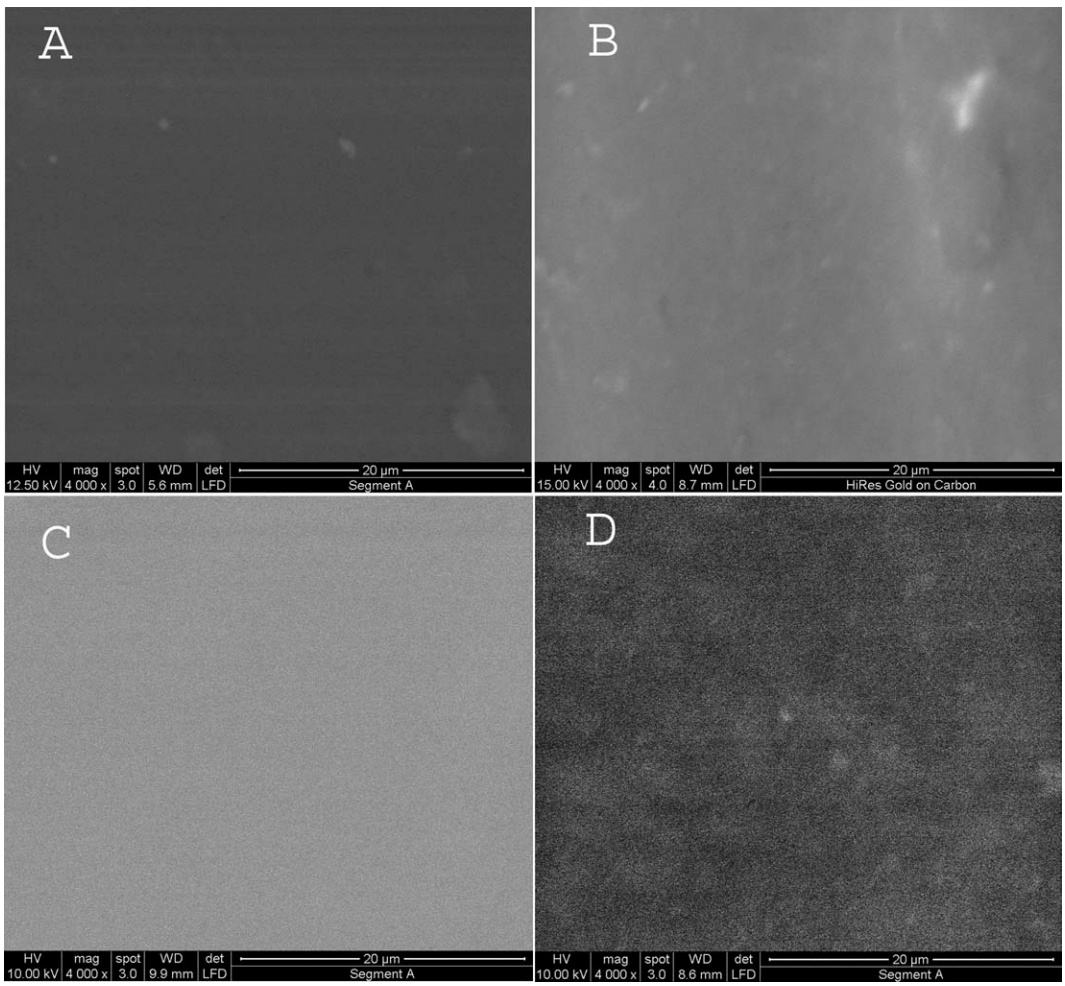

Figure 9 ESEM micrograph of (A) CH-0, (B) CH-V4, (C) CH-V6, and (D) CH-V10.

membranes do not allow the high molecular weight albumin to pass through them, which is highly beneficial for them to be proposed as possible hemodialysis membranes. Additionally, as reported earlier, the copolymers possess excellent mechanical stability, cytocompatibility and blood compatibility,

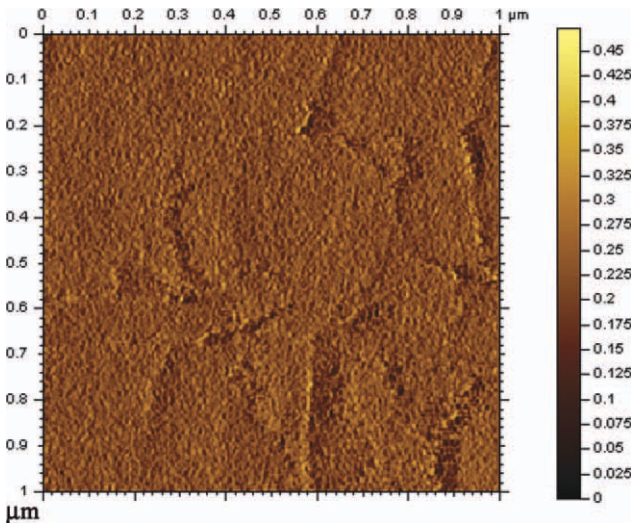

Figure 10 AFM image of a typical chitosan-*g*-PVAc membrane, CH-V6. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

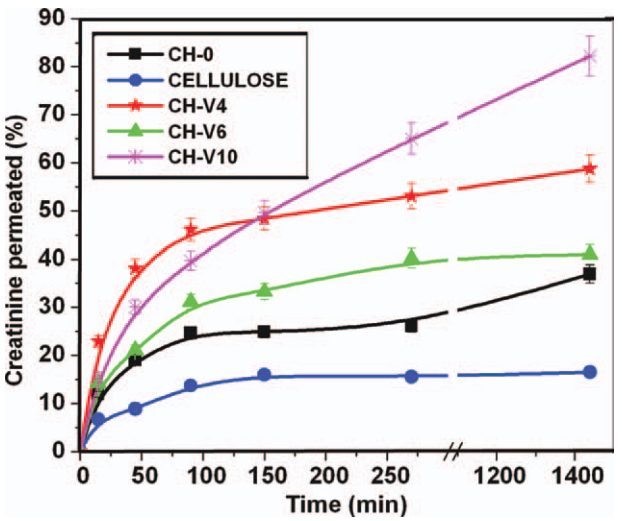

Figure 11 Permeation of creatinine through chitosan-*g*-PVAc membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

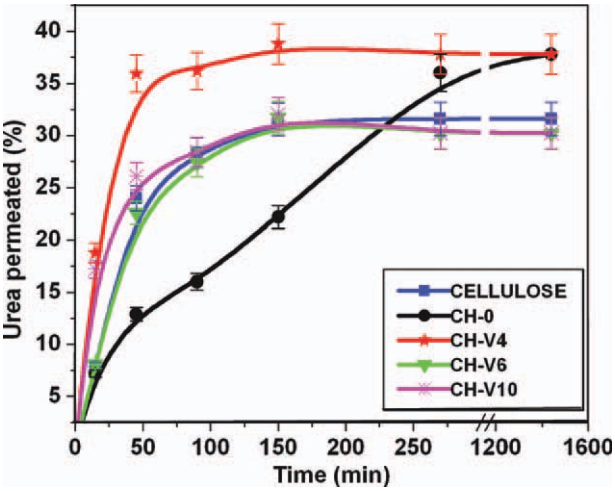

Figure 12 Permeation of urea through chitosan-*g*-PVAc membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

which further support their suitability for hemodialysis membrane applications.

When the amount of solutes permeated for a period of 24 h by each film is compared as shown in the Figure 15, all the copolymer films show better performance than both the cellulose and virgin chitosan films. Among the films, the composition having highest PVAc content, that is, CH-V10, shows the best performance.

CONCLUSION

Biocompatible and mechanically stable membranes could be synthesized from chitosan-*g*-PVAc copolymers. The permeability of the membranes for the low molecular weight solutes like urea, creatinine, and glucose were found to be superior to commercial cellulose films and were also proved to be impermeable to one of the essential nutrients, albumin. Hence, the need for the development of biocompatible, mechanically strong dialysis membranes could be addressed with the modification of chitosan through grafting with PVAc. Potential applications

TABLE VII
Permeation Coefficient of the Different Solutes Through Chitosan and the Chitosan-*g*-PVAc Membranes

Films	Creatinine ($P \times 10^5$ cm^2 min^{-1})	Urea ($P \times 10^5$ cm^2 min^{-1})	Glucose ($P \times 10^5$ cm^2 min^{-1})
CH-0	2.0	3.2	4.0
CH-V4	2.5	3.3	3.1
CH-V6	2.2	4.5	4.1
CH-V10	2.3	3.6	3.3
Cellulose	2.2	2.1	2.3

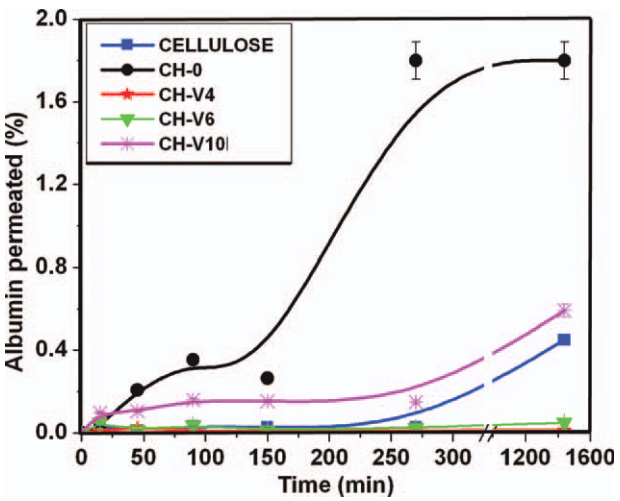

Figure 14 Permeation of albumin through chitosan-*g*-PVAc membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

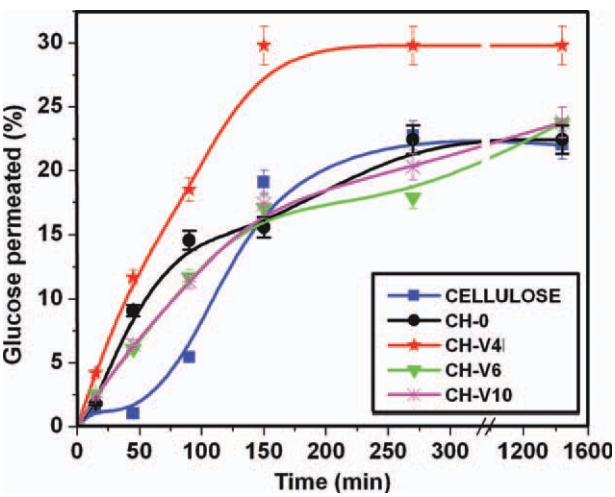

Figure 13 Permeation of glucose through chitosan-*g*-PVAc membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

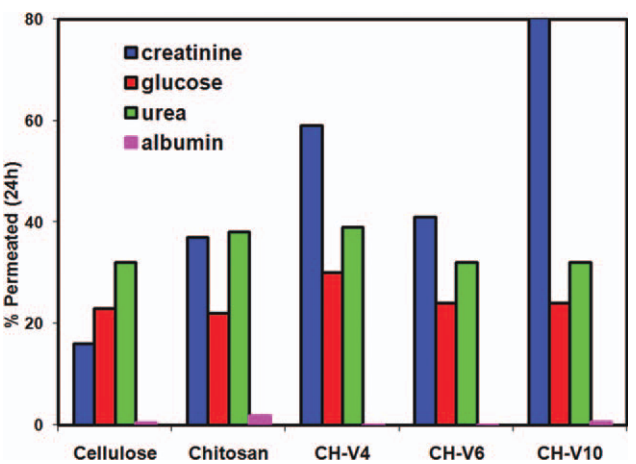

Figure 15 Comparison of percentage permeability of the solutes through chitosan-*g*-PVAc membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

like artificial kidney, artificial pancreas, and so forth, are envisaged from these membranes

The authors thank Director of Sree Chitra Tirunal Institute for Medical Sciences and Technology and Head of Biomedical Technology Wing for providing all the facilities and also Ms. L. Thomas for the AFM image and to "Kerala State Council for Science, Technology and Environment," for the funding.

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