HEMA-Grafted Chitosan for Dialysis Membrane Applications

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ABSTRACT: Chitosan was graft copolymerized with HEMA (2-Hydroxyethylmethacrylate) for the development of blood-compatible dialysis membranes. The permeation characteristics of HEMA-grafted chitosan films for four different solutes creatinine, urea, glucose, and albumin was studied *in vitro* at 37°C for assessment of the suitability as dialysis membranes. The grafted film CH-12.5 composition (425% grafting) showed very high permeation to creatinine by reaching the equilibrium within 45 min. The compositions CH-7.5 and CH-12.5 showed excellent permeation to glucose when compared to virgin chitosan films. In the case of urea permeation, all the grafted compositions exhibited higher percent permeation than the virgin chitosan films. The copolymer films CH-7.5 and CH-12.5 showed enhanced permeability for the high molecular weight solute, albumin. The other grafted copolymer compositions followed almost the same trend as that of chitosan for the low molecular weight solutes as well as the high molecular weight solute. The copolymer films were also found to be highly blood compatible, noncytotoxic, and biodegradable. Hence, the need for developing blood-compatible chitosan membranes with desirable permeability properties is achieved by the graft copolymerization of HEMA onto chitosan. J Appl Polym Sci 101: 2960–2966, 2006

Key words: chitosan; graft copolymer; permeability; blood compatibility; biodegradability

INTRODUCTION

Chitosan [β-(1-4)-2-amino-2-deoxy-D-glucose] is a copolymer of N-acetyl glucosamine and glucosamine units. Chitosan has very good properties as a biomaterial; it is biodegradable, biocompatible, nontoxic, and antithrombogenic.[1–3] These properties made chitosan widely applicable in the pharmaceutical and biomedical fields.

Chitosan has been reported as an artificial kidney membrane possessing good mechanical strength in addition to permeability to urea and creatinine. However, these membranes are impermeable to serum proteins. Thus, there is need to develop better hemodialysis membranes to provide greater selectivity and higher dialysis rates for medium- and large-size molecules.

Hemodialysis, an extracorporeal blood purification procedure, utilizes a polymeric membrane to remove desired amounts of solutes and water from blood. Commercially regenerated cellulose or cuprophan is used as artificial kidney membranes due to good solute permeability and mechanical strength.

Various modifications are suggested to dramatically improve the blood compatibility of chitosan membranes without altering its superior permeability. With the aim of preparing blood-compatible membranes without sacrificing the permeability of solutes, we synthesized chitosan modified with 2-hydroxyethylmethacrylate (HEMA). The method of synthesis and physicochemical characterization of the HEMA-grafted chitosan membranes have been reported earlier.[4] The copolymers showed good film-forming property. Transparent and tough films could be prepared by solution casting. It was of interest to evaluate the permeability of these materials for different solutes like urea, creatinine, glucose, albumin, etc.

EXPERIMENTAL

Materials

Chitosan was from India Sea Foods, Cochin; ceric ammonium nitrate was from E. Merck India, and used as such. 2-Hydroxy ethyl methacrylate (HEMA, Merck) was purified by vacuum distillation; albumin, creatinine (extra pure) glucose, and urea were pur-

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chased from SRL Pvt India Ltd., and were used as such.

Methods

HEMA-grafted chitosan copolymers were prepared by the method reported earlier.[4] Briefly, chitosan with varying extent of HEMA grafting were prepared. The different compositions were coded as CH-0, CH-5, CH-7.5, CH-10, and CH-12.5. The grafted product was dissolved in 2% aqueous acetic acid, and were cast into films and sterilized by ethylene oxide (ETO) method. It is reported that any of the sterilization methods like autoclave, ethyl alcohol, glow discharge, gamma irradiation, etc., can also be adapted for chitosan.[5]

Permeation studies

The permeation characteristics of the control and HEMA-grafted chitosan for four different solutes creatinine, urea, glucose, and albumin were studied *in vitro* at 37°C. Standard cellulose film was used for comparison. The experimental setup consisted of a two-chamber dialysis cell, in which 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, urea 37 mg/dL, glucose 16.5 mM/L, and albumin 8 g/dL), while the other chamber was filled with a solute-free buffer [phosphate-buffered saline (PBS) 7.4]. The cell was placed on a gentle mechanical shaker that 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 the picric acid method for creatinine, the DAM (Diacetyl monoxime) method for urea, the *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 equation

$$P = \ln (2\, C_0/C_t - 1) \times Vl/2a \qquad (1)$$

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 $\ln (2\, C_0/C_t - 1) \times Vl/2a$ versus time plots.

The actual amount permeated at time t, C_t, and percentage of initial amount permeated $C_t/C_0 \times 100$ was also calculated.

In vitro screening of materials

In this study, the effects of whole blood contact on WBC, RBC, platelets, plasma coagulation, and percentage hemolysis 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 a siliconized polystyrene Petri plate using a small drop of silicone adhesive and allowed to cure for 24 h. Empty wells were used as reference. Test materials were immersed in 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 counts of WBC, RBC, and platelets and also for total hemoglobin, percentage hemolysis, and coagulation analysis. The materials were left in the blood for 30 min under agitation at 75 ± 5 rpm using an Environ shaker thermostated at 37 ± 2°C. After 30 min, a 1-mL sample was again withdrawn for all the above studies.

Estimation of percentage hemolysis

The blood samples were centrifuged at 4000 rpm for 15 min and platelet poor plasma was aspirated. Plasma hemoglobin was measured in each sample by spectrophotometric analysis.

Estimation of coagulation parameters

The blood samples were centrifuged at 4000 rpm for 15 min and platelet-poor plasma was aspirated. Partial thromboplastin time was measured in each sample using a reagent kit obtained from Diagnostica Stago (France) on a start 4 Coagulation Analyzer.

Consumption of WBC, RBC, and platelets by cell counts

The count reduction was analyzed by detecting the counts in initial and 30-min samples using a hematology Analyzer Cobas Minos vet (Roche, France).

In vitro cytotoxicity test

L-929 fibroblast cells were used for the *in vitro* screening of chitosan and the modified chitosan films; 0.7 × 0.7-cm size pieces were used for the test. Test samples, negative controls (high-density polyethylene), and positive controls (copper) in triplicate were placed on a confluent monolayer of L-929 mouse fibroblast cells. After incubation of the cells with test samples at

TABLE 1
Permeation Coefficient of the Different Solutes through Chitosan and the HEMA-Grafted Chitosan Films

Films	Creatinine $P \times 10^5$ (cm^2/min)	Glucose $P \times 10^5$ (cm^2/min)	Urea $P \times 10^5$ (cm^2/min)
CH-0	1.9	4.0	3.2
CH-5	2.3	4.0	3.4
CH-7.5	2.4	2.4	4
CH-10	2.9	4.1	4.8
CH-12.5	2.7	2.5	3.9
Cellulose	2.2	2.3	2.1

37 ± 2°C for 24 ± 1 h, cell culture was examined microscopically for cellular response around the test samples. Cellular responses were scored as 0, 1, 2, and 3 according to noncytotoxic, mildly cytotoxic, moderately cytotoxic, and severely cytotoxic.

Biodegradation studies

The biodegradation studies of chitosan and the HEMA-grafted chitosan copolymers were carried out in enzyme solutions, namely trypsin in Tris buffer at pH 8.1 and papain in citrate buffer at pH 6. Samples were also maintained in Tris buffer pH 8.1, and Tris and citrate buffer at pH 6. Strips of dimensions 8 × 0.5 cm of chitosan, and the copolymers were immersed each in a separate tube containing 10 mL of the enzyme solutions (1% solution). Films of the same dimensions were put in 10 mL of buffer solutions also. All tubes were kept in an orbital shaker at 37°C. The weights of the strips were taken after the period of 3 days, 1 week, 2 weeks, and 1 month after washing with distilled water and drying in the oven.

RESULT AND DISCUSSION

Permeation studies

The design of artificial kidney systems has made possible repetitive hemodialysis and sustaining the life of chronic kidney failure patients. Chitosan membranes have been proposed as an artificial kidney membrane because of their favorable permeability and high tensile strength.[6–9] The most important part of the artificial kidney is the semipermeable membrane. Regenerated cellulose and cuprophane are the commercially available dialysis membranes. But, there is little selectivity in the separation of two closely related molecules.[10] Hence, novel membranes need to be developed for better control of transport, ease of formability, and inherent blood compatibility. Chitosan is said to be haemostatic, that is, aids in blood clotting, that can compromise the permeability characteristics.[11] So, attempts were made to improve the blood compatibility of chitosan membranes via surface modification with the least interference to their permeability properties.[12–15] A nonthrombogenic albumin-blended chitosan membrane was derived by immobilizing this bioactive complex via carbodiimide.[16] Reports are available regarding the permeability of various molecules through chitosan membranes,[16–18] which contain different immobilized and modified biomolecules. The permeation of creatinine, urea, glucose, and albumin through chitosan and HEMA-grafted chitosan membranes were studied in phosphate buffer 0.1*M* (pH 7.4) at 37°C.

The grafted film, CH-12.5 composition, showed very high permeation of creatinine reaching the equilibrium condition at 45 min. The maximum amount permeated is 30% of the initial concentration of creatinine taken in the donor compartment. All the other grafted compositions showed almost a similar trend in creatinine permeation as that of virgin chitosan, proving that grafting of HEMA onto chitosan did not change the creatinine permeability of chitosan membranes up to a certain level of grafting. After 150 min, all the films of chitosan and the grafted copolymers reached an equilibrium condition. The permeation coefficient value of the grafted films increases slightly as the concentration of HEMA increases (Table I). The permeation coefficient value of chitosan is 1.9×10^{-5}. The grafted film CH10 has a value of 2.9×10^{-5}. Figure 1 shows the percent permeation of creatinine versus time through chitosan and the HEMA-grafted chitosan films.

In the case of glucose permeation, the CH-12.5 composition showed excellent permeation properties when compared to virgin chitosan film. The CH-7.5 composition also showed enhanced percent permeability when compared to chitosan films. The other grafted copolymer compositions CH-5 and CH-10 fol-

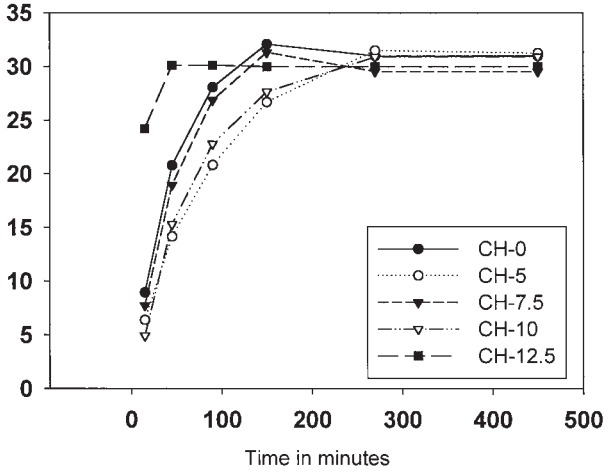

Figure 1 Permeation of creatinine through chitosan and the copolymer films.

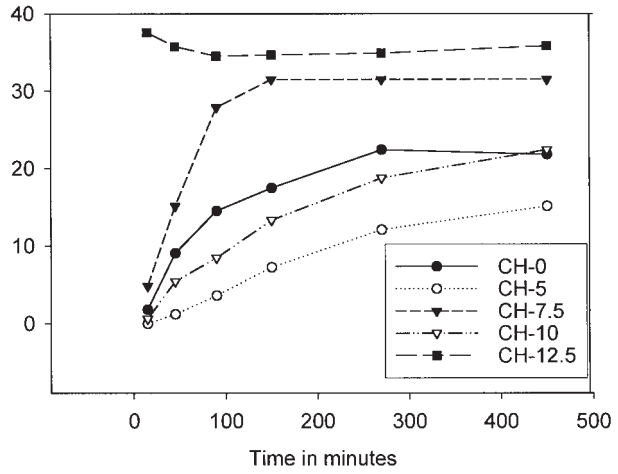

Figure 2 Permeation of glucose through chitosan and the copolymer films.

lowed almost the same trend as that of chitosan. Figure 2 shows the percent permeation of glucose versus time for chitosan and the HEMA-grafted chitosan films.

The grafted films showed higher percentage permeation for urea than virgin chitosan membrane (Fig. 3). The initial amount permeated by all the grafted films at 5, 15, 30, and 45 min is higher than that by the virgin chitosan membrane, showing an improved permeability for the HEMA-grafted chitosan films. The maximum amount permeated is the highest (i.e., 42.9%) for CH-12.5 composition. Hence, the HEMA-grafted chitosan films, especially the CH-12.5 composition, proved to be a good membrane for urea permeation also. Amiji[17] noted no significant increase in creatinine and urea permeability when chitosan films are surface modified with heparin. The permeability coefficient of the chitosan film is 3.2×10^{-5} cm^2/min, whereas all

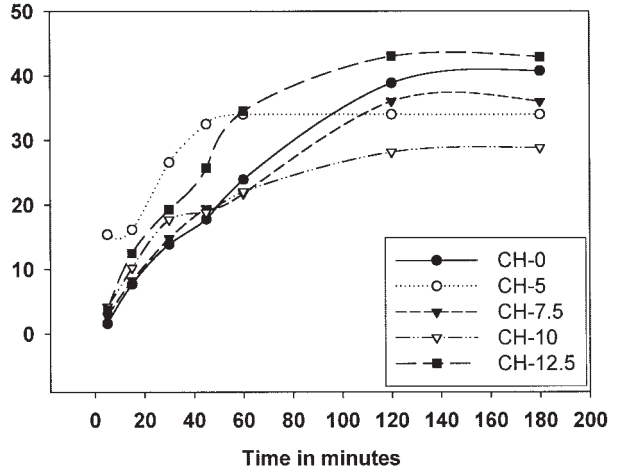

Figure 3 Permeation of urea through chitosan and the copolymer films.

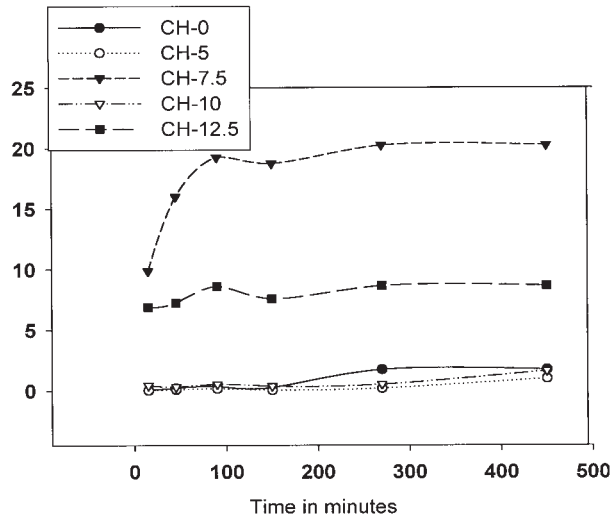

Figure 4 Permeation of albumin through chitosan and the copolymer films.

the grafted films showed higher permeation coefficient value (Table I).

Chitosan permeated a maximum of 1.8% of the initial amount total albumin. The grafted copolymers CH 7.5 and CH-12.5 showed higher level of permeation Figure 4. The HEMA-grafted chitosan compositions, CH-5 and CH-10, showed the same trend as that of chitosan.

As the hydrophilicity is increased by graft copolymerization of HEMA onto chitosan as is evident from swelling studies and contact angle studies,[4] the permeability of solutes through the membranes prepared by the copolymers gets enhanced. A similar trend has been reported for chitosan modified with water soluble polymers like PEG and PVA.[18]

In vitro screening of materials

The RBC and WBC counts (initial and after 30 min of contact of the materials with blood) are given in the Tables II and III. From the data, it is clear that none of the materials caused a reduction in the cell count as an

TABLE II
RBC Count Initial and After 30-Min Contact of the Materials with Blood

Material	Total cell number initial ($\times 10^9$)	Total cell number initial after 30-min exposure ($\times 10^9$)
CH-0	6.81	6.12
CH-5	6.01	6.16
CH-7.5	6.25	6.16
CH-12.5	6.21	6.3
Reference material	6.21	6.6

TABLE III
WBC Count Initial and after 30-Min Contact of the Materials with Blood

Material	Total cell number initial ($\times 10^6$)	Total cell number initial after 30-min exposure ($\times 10^6$)
CH-0	11.1	13.05
CH-12.5	11.85	14.1
CH-10	11.7	12.75
CH-5	12.15	12.0
Reference material	11.7	12.9

effect of contact with the test materials. The total number of platelets in the exposed blood is given in Table IV. When compared to the reference material, the test samples do not cause a significant change in the platelet counts of the blood samples to which they are exposed. The hemolysis percent also was unaffected due to material contact. Hence, the *in vitro* screening of the materials shows that the HEMA-grafted chitosan films are suitable for any type of blood contacting applications.

In vitro cytotoxicity test

L929 is an established and well-characterized mammalian cell line that has demonstrated reproducible results. One of the most sensitive toxicity testing protocol is based on the direct contact of the sample with the cell culture. Assessment of the cytotoxicity of the membranes was carried out according to ISO 10993-5, 1999. The fibroblast cells are spindle shaped, and were evaluated for general morphology, vacuolization, detachment, cell lyses, and degeneration when in contact with the materials. The L929 cells in contact with test samples retained their spindle-shaped morphology when compared to negative control (high-density polyethylene). The membranes also did not induce deleterious effects such as detachment, degenerative, and lysis with L929 fibroblasts when placed directly on the monolayer of the cells. Figure 5(a, b) show the chitosan and the HEMA-grafted chitosan films when exposed to L929 fibroblast cells. Figure 5(c) shows the morphology of normal fibroblast cells.

TABLE IV
Platelet Count Initial and after 30-min Contact of the Materials with Blood

Material	Platelet count—initial ($\times 10^8$)	Platelet count—30 min ($\times 10^8$)
CH-0	3.54	3.04
CH-12.5	4.18	3.15
CH-10	4.14	3.09
CH-5	3.93	3.15
Reference material	4.02	2.70

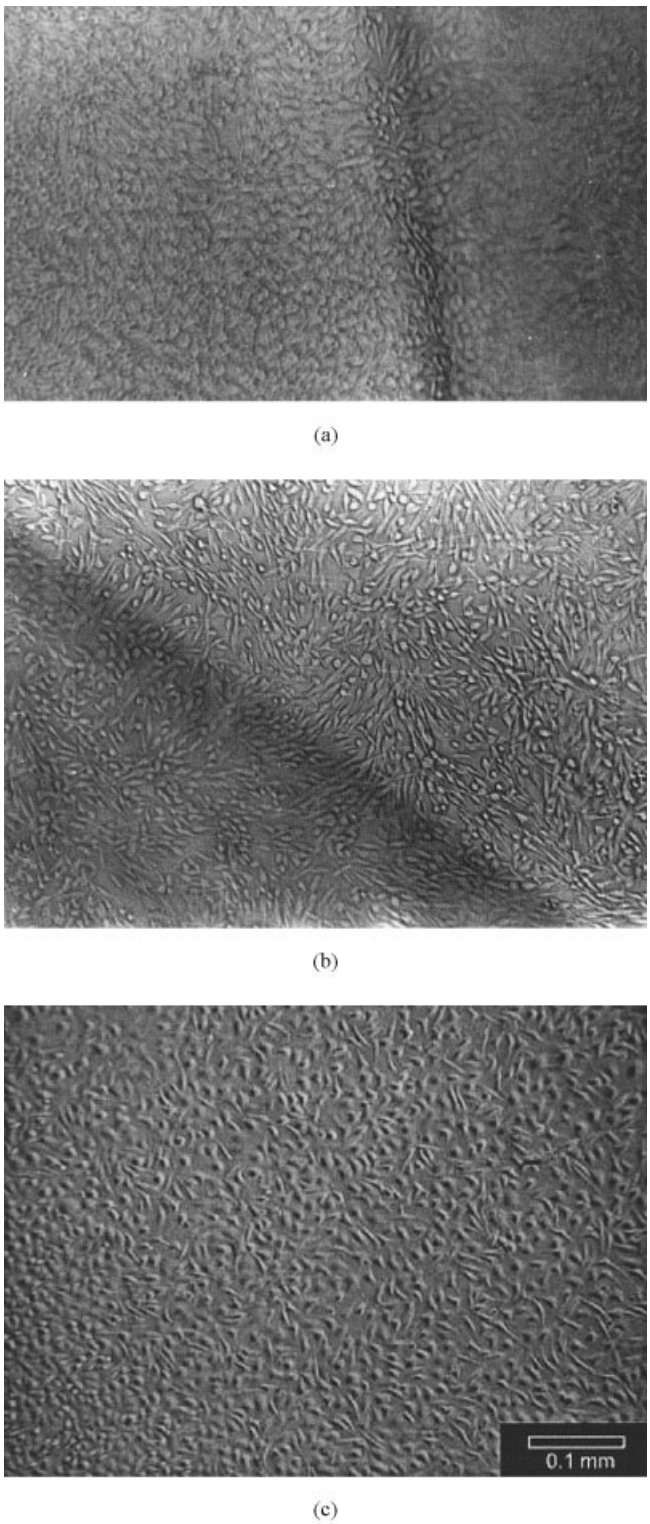

Figure 5 (a) Viable fibroblast cells in contact with chitosan. (b) Viable fibroblast cells in contact with HEMA grafted chitosan. (c) Normal L929 fibroblast cells.

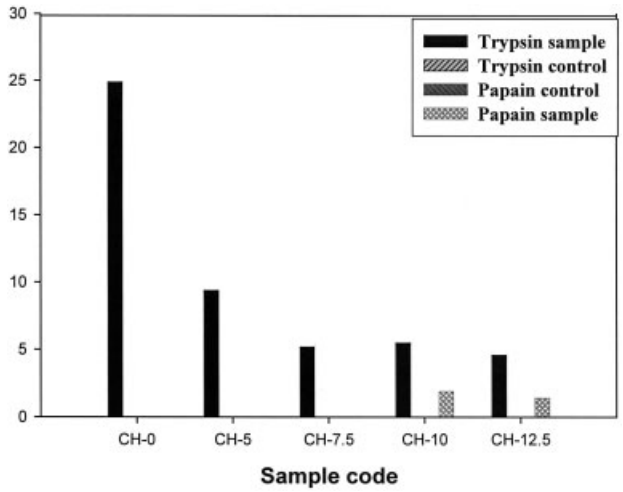

Figure 6 Percent loss of weight of chitosan and the copolymer films after keeping in enzyme solution for 1 month.

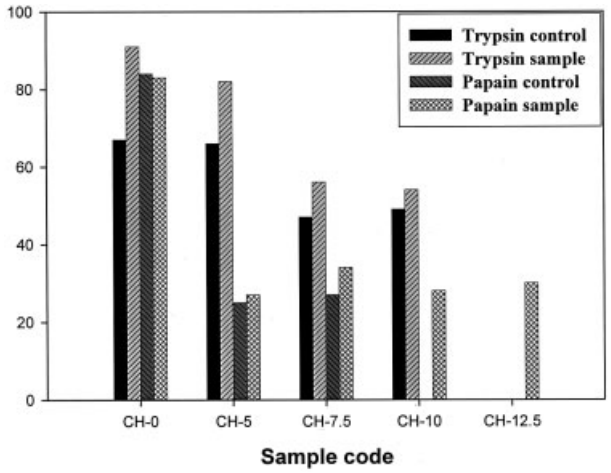

Figure 7 Percent loss of mechanical properties of chitosan and the copolymer films after keeping in enzyme solution for 1 month.

Biodegradation studies

In vitro biodegradation studies of the films are carried out in enzyme solutions like trypsin, papain, etc. The biodegradability of the films is assessed by checking the mass loss, FTIR spectra, and the mechanical properties of the films. Figure 6 gives mass of the different films after biodegradation. The films kept in trypsin solution only have undergone mass loss. We can see that the chitosan films have a higher percent mass loss than the grafted ones. As the percent grafting increases to a certain extent, the percent mass loss becomes constant. Hence, grafting can have a control over the biodegradability of chitosan. In other words, the grafted films can remain in the enzymatic environment for a more prolonged time than that of virgin chitosan films.

Figure 7 shows the percent loss of stress of chitosan and the grafted films after being in the test solutions. The films kept in trypsin solution have undergone maximum degradation. For chitosan films, all the test solutions (buffers and enzyme solutions) caused degradation. But the effect decreases as the extent of grafting enhances. The CH-12.5 films in which the grafting percent is the maximum, has the minimum loss of mechanical property. Figure 8 shows the FTIR spectra of chitosan films before and after biodegradation. The decrease in peak intensities of the 1593 and 1420 cm^{-1} peaks in the chitosan films are indicative of the deterioration of the —NH_2 groups and —CH_2 bonds in the chitosan chains. Figure 9 is the FTIR-ATR spectrum of the HEMA-grafted chitosan films before biodegradation and after keeping the films in enzyme solution (Trypsin) for 1 month. The spectra clearly confirm that degradation has taken place in the copolymer films when they are exposed to an enzymatic environment. As an effect of grafting HEMA on chitosan the biodegradability of chitosan can be controlled.

Thus, grafting with synthetic polymers improves the stability of chitosan films in the enzymatic environment without sacrificing its blood compatibility or noncytotoxicity. We also proved that the permeability of all the grafted films are unaffected due to grafting, and in certain compositions grafting resulted in better permeability in comparison to virgin chitosan. All the compositions showed higher percent permeation for the solutes than that for standard cellulose films. Table I shows the permeation coefficient values of chitosan and the HEMA-grafted chitosan films compared with the standard cellulose film. We can see that all the films showed better permeability than that of standard cellulose films.

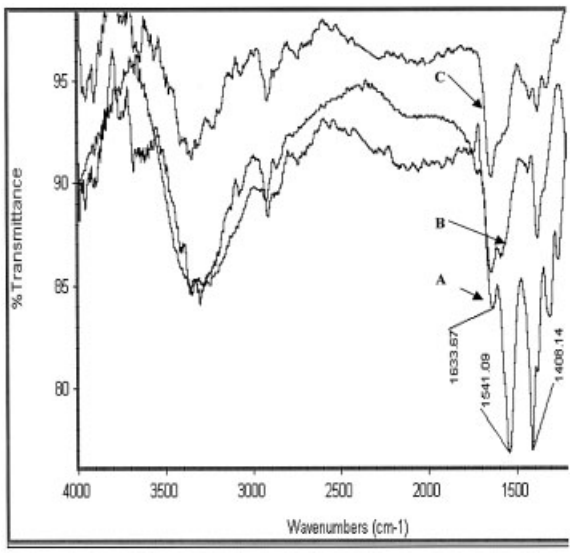

Figure 8 FTIR Spectra of (A) Chitosan before biodegradation, (B) chitosan in Tris buffer, (C) chitosan in trypsin.

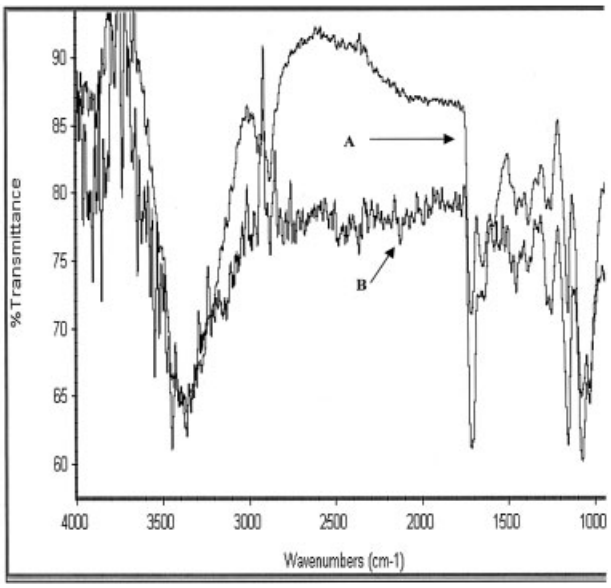

Figure 9 FTIR Spectra of (A) HEMA-grafted chitosan in Tris buffer, (B) HEMA-grafted chitosan in trypsin.

CONCLUSIONS

The HEMA-modified chitosan films were studied for their suitability to be applied for dialysis membranes. All the compositions were found to be highly blood compatible and noncytotoxic to rat fibroblast cells. The biodegradability of virgin chitosan could be controlled by way of HEMA grafting. The high biodegradability of the chitosan films limits its membrane type applications, whereas HEMA-grafted chitosan films show less degradability and has proved to be blood compatible, which makes them promising materials for dialysis membranes. The grafted membranes showed better permeability for the solutes like glucose, urea, and creatinine in comparison to the virgin chitosan and even standard cellulose films.

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