Graft Copolymerization of 2-Hydroxyethylmethacrylate onto Chitosan Films and Their Blood Compatibility

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SYNOPSIS

This article examines the blood compatibility of chitosan grafted with 2-hydroxyethylmethacrylate (HEMA) using ^{60}Co gamma radiation. The effect of various syntheses conditions on the grafting was determined. The solvent composition has a marked effect on the degree of grafting. Maximum yield was obtained in an equivolume mixture of a water–methanol system. The percent grafting increased with the increase in the monomer concentration up to 20 vol % in a total dose of 0.216 Mrad. The percent grafting was found to be higher at a lower dose rate for a constant total dose of 0.216 Mrad. The swelling of grafted chitosan films decreased on increasing graft level. The grafted copolymers showed improved blood compatibility as compared to original chitosan sample. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Chitosan [(1 → 4)2-amino-2-deoxy-D-glucan] is a polyaminosaccharide obtained from chitin by a deacetylation process.[1] It is a copolymer of *N*-acetylglucosamine and glucosamine units and, generally, contains more than 90% glucosamine units. Several attempts have been made to use this material for biomedical applications. It has been reported that this biomolecule, which is nontoxic, biodegradable, and biocompatible, has applications as an anticoagulant, a wound healing accelerator, and in drug delivery systems.[2] Hirano and Noishiki[3] found that *N*-acetyl and *N*-hexanoyl chitosan membranes are less thrombogenic than chitosan membrane.

Recently, chemical modifications of chitosan through grafting has received considerable attention in the area of biomedical applications. Several studies on graft copolymerization onto chitin and chitosan have been reported using various chemical initiators like azobisisobutyronitrile (AIBN),[4] iron(II)-hydrogen peroxide,[5] tributylborane,[6] and cerium.[7] These initiators are known to be toxic for living systems. In the present work, attempts were made to improve the blood compatibility of chitosan by grafting it with 2-hydroxyethylmethacrylate (HEMA) using gamma radiation because this method leaves no toxic impurities in the system.

EXPERIMENTAL

Materials

Chitin, extracted from Prawn shell was obtained from the Central Institute of Fisheries Technology, Cochin, India. HEMA monomer (E. Merck, Germany) was distilled at 70°C/20 mmHg and stored at 4°C.[8] Methanol (A. R. Grade) was obtained from BDH Chemicals, India.

Radiation Source

The irradiation of samples was carried out in a ^{60}Co gamma radiation chamber supplied by Bhabha Atomic Research Centre, Bombay, India.

Preparation of Chitosan

Chitosan was prepared by deacetylation of chitin using 50 wt % NaOH at 110°C for 3 h. After this

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process, flakes separated from the alkali layer were extensively washed with distilled water to remove the traces of alkali. The resulting flakes were dried in a vacuum oven at 50°C for 72 h. Chitosan flakes were then dissolved in a 1% aqueous acetic acid solution and filtered through a sintered glass filter (porosity 1). The resulting solution was regenerated into a 10% NaOH solution. The precipitate were washes several times with distilled water to remove the traces of alkali. Finally, chitosan in powder form was obtained by freeze drying. The powdered chitosan was purified using the method given by Muzzarelli et al.[9] by extracting it in a Soxhlet extractor with methanol, water, petroleum ether, and acetone in that order, each for about 24 h. The degree of deacetylation of the purified chitosan was 83% and the viscosity average molecular weight was around 2.5×10^5 Da.

Chitosan films were prepared by dissolving the material in 1% acetic acid solution and subsequently spreading it on a glass plate maintained at room temperature in a dust free environment for 72 h, followed by immersing it in a 10% aqueous sodium hydroxide solution. The thickness and area of all chitosan films under study were kept constant.

Grafting Procedure

Graft copolymerization was carried out in standard joint borosil glass tubes of 13×2.8 cm size. All samples were kept in glass tubes for equilibrium swelling in water–methanol solvent for 20 h before grafting. HEMA was added just before the irradiation of samples. The monomer concentration was kept at 10 vol % in most of the experiments, unless otherwise specified. The system was deoxygenated by slow bubbling of nitrogen gas through the solution for 5 min in an ice bath. The sample tubes were irradiated for a specified time in the gamma radiation chamber. After irradiation, the reaction was stopped by the addition of a large volume of methanol to the reaction mixture in the tubes. The homopolymer formed during reaction was removed by Soxhlet extraction in methanol for 24 h. All samples were then dried under vacuum at 50°C until a constant weight was attained. The dry weight of grafted samples was measured and the percentage of grafting was calculated using the following relationship:

$$\% \text{ grafting} = \frac{W_2 - W_1}{W_1} \times 100$$

where W_1 = weight of original sample and W_2 = weight of grafted sample.

Swelling Studies

The swelling studies of original and modified chitosan films were carried out in phosphate buffer solution (pH 7.4, 0.1 *M*) at 37°C for 24 h. The samples were then removed and blotted for approximately 30 s with the absorbent paper to remove the surface water. The percent swelling was calculated as the weight increase with respect to the weight of dry sample using the following relationship:

$$\text{percent swelling} = \frac{W_s - W_d}{W_d} \times 100$$

where W_s = weight of swollen sample and W_d = weight of dry sample.

Blood Compatibility Studies

Blood compatibility of various chitosan samples were evaluated by the following methods.

Thrombus Formation Studies

Antithrombogenic properties of original and modified chitosan samples were measured with human acid citrate dextrose (ACD) blood using the kinetic method developed by Imai and Nose.[10] All samples before this test were hydrated in saline water at 37°C for 24 h in a constant temperature water bath. ACD blood (0.3 mL) was added to each sample. The reaction was initiated by the further addition of 0.02 mL of 0.1 *M* calcium chloride solution to each sample. After 30 min, distilled water (4.0 mL) was added to stop the reaction and the thrombus formed was separated from it by soaking in water for 10 min at room temperature and then fixed in 36% formaldehyde solution (2.0 mL) for another 10 min. This fixed clot was placed in water for 10 min and after drying its weight was measured.

Hemolysis Assay

Chitosan films (1 cm^2) were equilibrated in normal saline water for 60 min at 37°C and human ACD blood (0.25 mL) was added on films. After 20 min, 2.0 mL of 0.9% sodium chloride (NaCl) saline was added to each sample to stop hemolysis and the samples were incubated for 60 min at 37°C. Positive and negative controls were obtained by adding 0.25 mL of human ACD blood and 0.9% NaCl saline, respectively, to 2.0 mL of double distilled water. Incubated samples were centrifuged for 45 min, the supernatant was taken, and its absorbance was re-

corded on a spectrophotometer (model UNIKON 930) at 545 nm. The percent of hemolysis was calculated using the following relationship:

$$\% \text{ hemolysis} = \frac{A \text{ test sample} - A\ (-) \text{ control}}{A\ (+) \text{ control} - A\ (-) \text{ control}} \times 100$$

where A = absorbance. The absorbance of positive and negative controls was found to be 3.44 and 0.04, respectively.

Scanning Electron Microscopic (SEM) Studies

Original and modified chitosan samples were resuspended in human ACD blood for 40 min. All samples were fixed in 10% formaldehyde solution. After washing in distilled water and drying in successive ethanol–water gradients, the samples were finally treated with absolute ethanol. The dried samples were mounted on a metal stub. Silver coating approximately 100-Å thick was achieved on the samples in a vacuum evaporator. All samples were scanned on an SEM Stereoscan A4 (Cambridge Scientific Instrument Ltd, England) system.

RESULTS AND DISCUSSION

During grafting studies it was found that the degree of grafting varied up to 5–8% for the same grafting experiment on different days, but the trend of grafting curves was reproducible.

The increase in weight of extracted copolymer samples, as compared to the weight of original chitosan, along with their infrared spectra revealed the evidence of grafting. The Fourier-transform infrared (FTIR) spectra of original and grafted chitosan are shown in Figure 1. The spectra exhibited a band at 1665 cm^{-1} that may be due to carbonyl absorption of chitosan and the appearance of a new peak at 1725 cm^{-1} may correspond to the carbonyl absorption of grafted poly(HEMA) chains. Two other bands in the FTIR spectrum of grafted chitosan, at 825 and 755 cm^{-1}, were also observed. They were assigned to rocking absorption of $-CH_2$ groups[11] contained in poly(HEMA) chains.

Effect of Solvent Composition

The effect of solvent composition on the grafting of HEMA onto chitosan film is shown in Figure 2. It may be observed that the percentage grafting in pure water as well as pure methanol is low (< 5%). The addition of methanol in water had a marked effect on the degree of grafting of HEMA onto chitosan. The percentage grafting increases with the increase of methanol content in the water–methanol mixture and a maximum grafting (52%) was observed in 1 : 1 water–MeOH system. This may be because of the formation of homopolymer during graft copo-

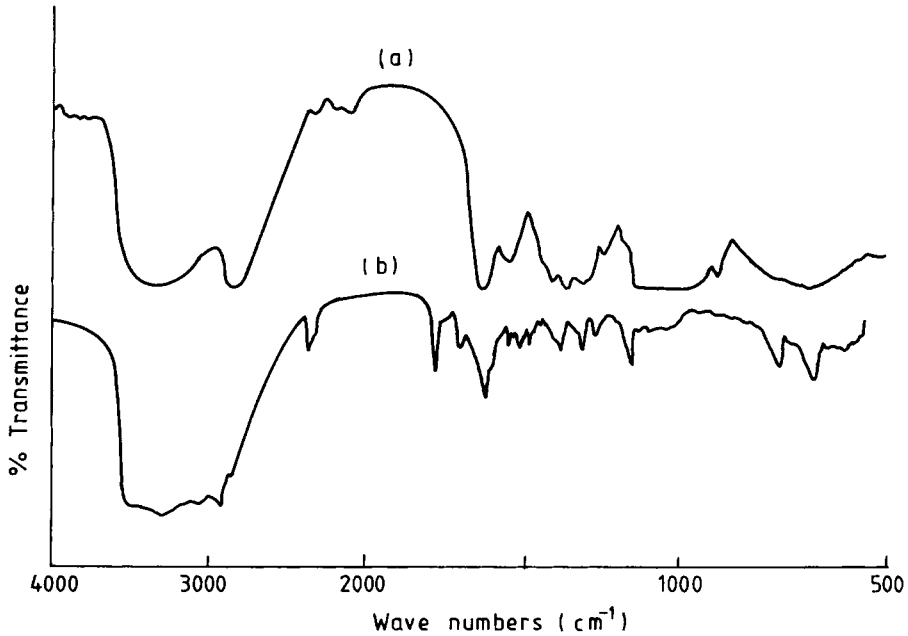

Figure 1 FTIR spectra of (a) chitosan and (b) grafted chitosan films with HEMA (graft level 14%).

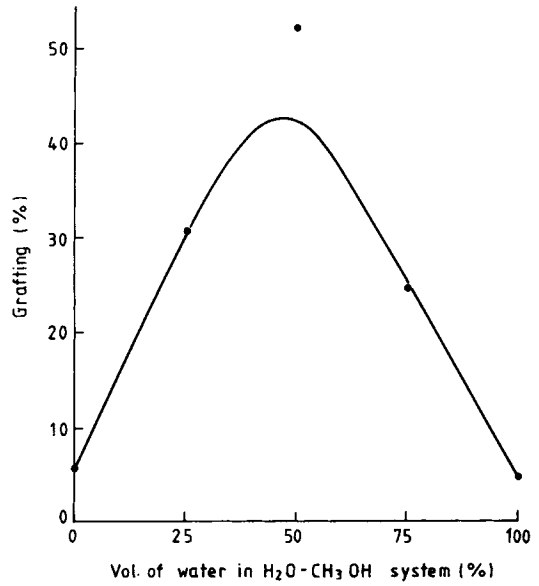

Figure 2 Effect of solvent composition on the grafting. Concentration of HEMA 10 vol %; dose rate 90 rad/s; total dose 0.216 Mrad.

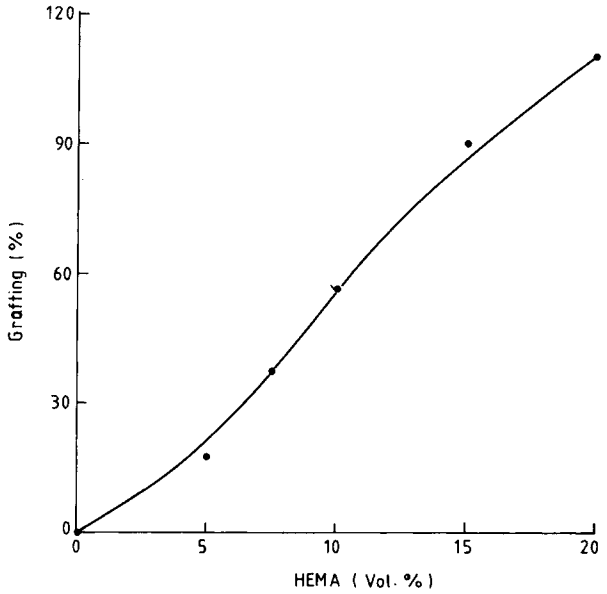

Figure 3 Effect of HEMA concentration on the grafting. Dose rate 90 rad/s; total dose 0.216 Mrad; solvent water–methanol (1 : 1).

lymerization of HEMA onto chitosan remains in solution which increases the viscosity of the solution and subsequently reduces the migration of HEMA onto the chitosan surface. The addition of methanol reduces the homopolymerization and retards the viscosity of the solution. These results reveal the importance of water for the grafting of HEMA onto chitosan. In grafting it appears, therefore, that the presence of water is responsible for the rupture of intermolecular hydrogen bonds in chitosan molecules, as a consequence of which HEMA molecules can diffuse readily to them. A similar behavior was also reported by Shigeno et al.[12] for chitin. The percentage of grafting decreases on increasing methanol content beyond 50% in methanol–water system. The higher content of methanol in the solvent mixture appears to be an inhibitor not only for grafting but also for the homopolymerization.

Effect of Monomer Concentration

The relation between percent grafting and monomer concentration is shown in Figure 3. Grafting was carried out at room temperature at a dose rate of 90 rad/s for a total dose of 0.216 Mrad in water–methanol (1 : 1) mixture. The results showed that there is an increase in the percent grafting with increasing monomer concentration. During the grafting process, monomer continuously diffuses into the polymer matrix. The ability of chitosan macroradicals to capture HEMA depends on the availability of HEMA molecules in their vicinity. Therefore, the increase in monomer concentration leads to an increase in graft content.

Effect of Dose Rate

Grafting was studied at different dose rates (viz. 22.5, 45.0, and 90.0 rad/s) in water–methanol (1 : 1) system. The results are depicted in Figure 4. At

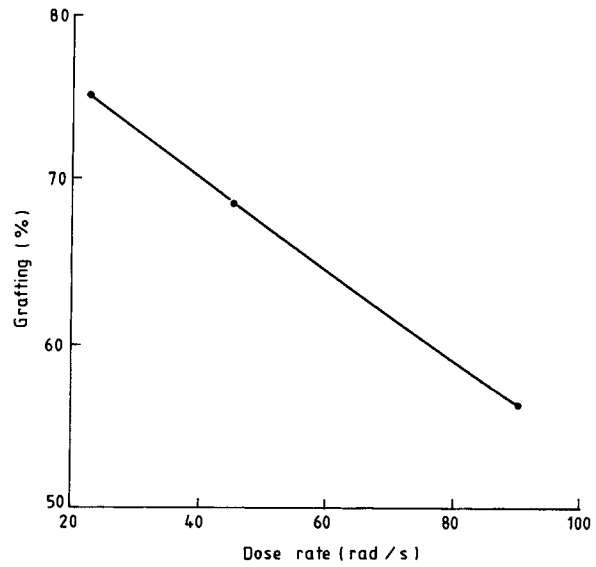

Figure 4 Effect of dose rate on the grafting. Concentration of HEMA 10 vol %; total dose 0.216 Mrad; solvent water–methanol (1 : 1).

a constant total dose of 0.216 Mrad, grafting was found to be higher (76%) at lower a dose rate (22.5 rad/s) for chitosan samples. Similar behavior has been reported for other systems.[13] It has been reported that during irradiation a number of free radical species are formed and their number increases with an increase in dose rate.[14] The monomer and radical species may react either with the polymer backbone (grafting) or with each other (homopolymerization).[15] During the graft copolymerization process, monomer molecules continuously diffuse into the polymer matrix at a constant rate irrespective of irradiation dose rate. Under such conditions the greater availability of free radicals at the higher dose rate increases the rate of homopolymerization. As a result of this the relative rate of grafting decreases appreciable at a higher dose rate.

Effect of Total Dose

The effect of total dose on grafting is presented in Figure 5. A total dose up to 0.54 Mrad was used at a dose rate of 90 rad/s. The graph exhibited a linear increase in percent grafting with the increase in dose of irradiation up to 0.324 Mrad; beyond this there is a marked decrease in the rate of grafting and tendency to level off. During the graft copolymerization reaction, the availability of monomer is higher in the initial stages and, hence, the monomer can diffuse very easily to the grafting sites and grafts readily onto the chitosan film. The rate of homopolymer formation does not have much affect on grafting kinetics. Both homopolymerization and copolymerization reactions proceed simultaneously until a total dose of 0.324 Mrad. Beyond this limit a decrease in the percent grafting results in the increase of the rate of homopolymerization as compared to the rate of grafting. The increasing content of grafted poly(HEMA) acts as a barrier against the diffusion of monomer into the polymer matrix resulting in the increase of the rate of homopolymerization and a decrease in the graft content.

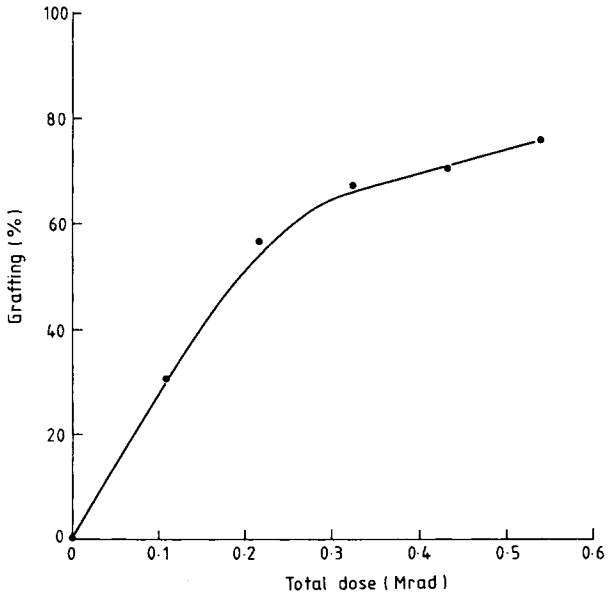

Figure 5 Effect of total dose on the grafting. Concentration of HEMA 10 vol %; dose rate 90 rad/s; solvent water–methanol (1 : 1).

Table I Results of In Vitro Blood Compatibility of HEMA-Grafted Chitosan Films

Sample No.	Grafting (%)	Swelling (%)	Thrombus Formed (mg)	Hemolysis (%)
C-0[a]	—	110	189	5.59
C-1[b]	—	108	191	5.38
C-2	14	89	161	1.28
C-3	29	78	143	0.96
C-4	37	71	128	0.73
C-5	58	65	117	0.50
C-6	108	58	120	0.46

Results are the average of three experiments.
[a] Original.
[b] Irradiated.

Swelling Studies

The swelling behavior of chitosan samples is presented in Table I. It is observed that original chitosan film swells up to 110% and there is no significant change in the swelling of irradiated chitosan film. The swelling of chitosan films after grafting with HEMA decreases up to 58% at 108% graft level. This decrease may be due to cross-linking of HEMA, in bulk, of chitosan film and blocking of the free amino groups on the film surface by HEMA molecules.

Blood Compatibility

Thrombus Formation

The weight of the thrombus formed by different chitosan samples are listed in Table I. The weight of the clot formed is maximum for original chitosan film but there is no significant change in the weight of the thrombus formed on the irradiated chitosan

sample. The formation of a thrombus on chitosan films decreases after grafting with HEMA. In spite of the hydrophilic nature of chitosan, it is thrombogenic due to the presence of free amino groups. The free amino groups may form polyelectrolyte complexes with acidic groups of cellular elements of blood.[16] Grafting of HEMA on amino groups might lead to the creation of hydrophilic and/or hydrophobic microdomains that retard thrombus formation and consequently improve blood compatibility.[17]

Hemolysis Assay

Hemolytic activity of all chitosan samples are presented in Table I. The percentage of hemolysis is maximum (100%) for a distilled water added blood sample (+ve control). For the original chitosan sample, maximum hemolysis was observed; however, it decreases in all the grafted samples. This might be due to the change in surface composition because of grafting and the blocking of reactive free amino groups by HEMA molecules.

SEM

SEM micrographs of original and grafted chitosan samples after exposure to blood are shown in Figures 6 and 7. Figure 6 is the SEM micrograph of red blood cells exposed to original chitosan film and shows their deformation and the coagulum formation. Figure 7 revealed that there is a significant reduction in the coagulum formation on grafted chitosan film as compared to original chitosan film.

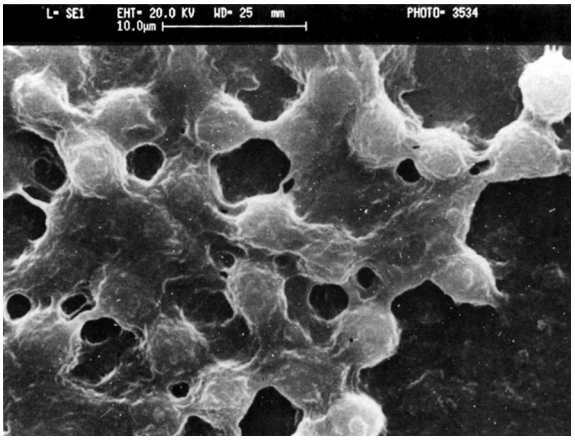

Figure 6 SEM micrograph of original chitosan after exposure to blood.

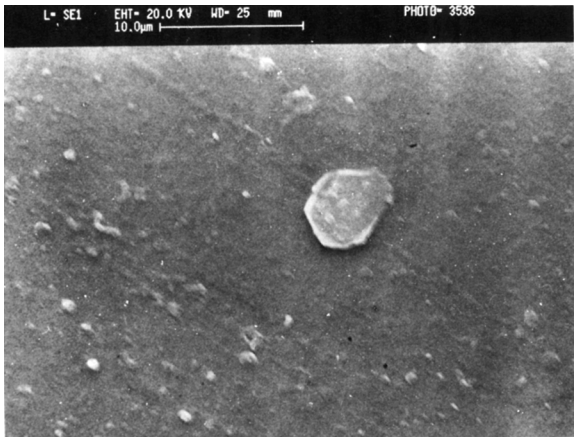

Figure 7 SEM micrograph of grafted chitosan with HEMA after exposure to blood (graft level 14%).

CONCLUSION

It can be concluded that the blood compatibility of chitosan can be improved by grafting with HEMA. It has also been found that the level of grafting can be controlled by appropriate grafting conditions, namely, solvent composition, monomer concentration, dose rate, and total dose.

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