Surface Modification of Cellulose Filter Paper by Glycidyl Methacrylate Grafting for Biomolecule Immobilization: Influence of Grafting Parameters and Urease Immobilization

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ABSTRACT: Graft copolymerization of glycidyl methacrylate (GMA) onto cellulose filter paper (CFP) was carried out by a free-radical initiating process using ceric ammonium nitrate (CAN) as an initiator. Optimum conditions pertaining to different grafting percentages were evaluated as a function of monomer and initiator concentrations, polymerization time and temperature. CFP with various graft levels of GMA was characterized by fourier transform infrared (FTIR) spectroscopy and thermo gravimetric analysis (TGA). Surface morphology of ungrafted and grafted CFP was evaluated by scanning electron microscopy (SEM). Attenuated total reflectance (ATR)-FTIR spectral analysis provided the evidence of grafting of GMA onto CFP. The maximum grafting of 102% was achieved by using 4×10^{-3} molL^{-1} CAN and 5% of GMA (w/v) monomer at 60°C in 25 min. The CFP-g-GMA surfaces with different graft levels were evaluated as a support for immobilization of biomolecules. Urease was selected as the model enzyme to be covalently coupled through the surface epoxy groups of the CFP-g-GMA discs. Immobilized discs were further studied for urea estimation and their reusability. Although the highest degree of urease immobilization was observed at 100% (162-μg urease/disc) graft level, the urease immobilized on discs with 70% (105-μg urease/disc) graft level gave the maximum activity of the enzyme. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 1381–1390, 2009

Key words: ceric ammonium nitrate; glycidyl methacrylate; chemical grafting; urease immobilization; cellulose filter paper

INTRODUCTION

A range of synthetic and natural polymers such as, polypropylene, polystyrene, nylon, chitosan, and cotton have long been investigated for their use as matrix and support material.[1–5] But in recent years there has been considerable interest and stress on the use of biodegradable polymers after suitable modification to have potential end use. Filter paper, a form of cellulose—a natural carbohydrate polymer—is an inexpensive, easily available, degradable, and renewable biopolymer with very good mechanical properties. However, its application in several technologically important fields is limited because of the lack of reactive functional sites and thus the desirable surface properties. The use of cellulose-based materials could thus be extended to new areas by altering and tailoring its chemical and physical properties through the incorporation of functional moieties onto its surface. Grafting is one of the promising and attractive methods to introduce a variety of functional groups to a polymer.[6] Of the various techniques used for grafting, the two most widely used are photoinitiated and chemical-initiated grafting and the latter has generally been reported to give higher graft levels.[7] Chemical grafting of various monomers such as acrylamide on polyethylene oxide and gum, sulfo ammonium zwitterions monomer on poly(ether urethane) and methyl methacrylate (MMA) onto silk sericin has efficiently been achieved by using ceric ammonium nitrate (CAN) as an initiator.[8–11] Glycidyl methacrylate (GMA) grafted surfaces have been used for covalent coupling of biomolecules like enzyme at both laboratory and industrial scale.[12] Epoxide groups directly react with sulfhydryl, amino and carboxyl groups to form stable covalent bonds with biomolecules.[13–19] Such epoxy-modified polymer surfaces are stable during long storage periods and resistant against hydrolysis.[20]

Immobilization of biomolecules onto the matrix is generally carried out by means of entrapment, physical adsorption, copolymerization, or covalent attachment.[21] Biomolecules covalently bounded to the support show better shelf life and operational stability as compared with the nonspecifically bound

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molecules (by adsorption), which leak out during storage and/or assay. Enzyme immobilization has been subject of attention because of the limitation of the use of soluble enzyme due to their high cost, availability in small amounts, instability, and the limited possibility of economic recovery from a reaction mixture. Urease (EC 3.5.1.5), a nickel-dependent metalloenzyme, catalyzes the hydrolysis of urea to form ammonia and carbon dioxide.[22]

$$\text{Urea} + H_2O \rightarrow CO_2 + 2NH_4^+$$

The immobilized urease has a broad application spectrum. It can be used in biomedical application for blood detoxification or dialysate regeneration system for artificial kidney, in food industry for removal of traces of urea from beverages and foods, in agriculture for effluent treatment, and in biological fluids, such as blood, urine, etc and water, for analytical determination of urea.

The aim of this investigation is to develop a process of imparting effective functional groups onto cellulose filter paper (CFP) support using GMA as a monomer and CAN as a chemical initiator, with the view that the reactive functional groups introduced to the surface will provide covalent attachment to the urease and the enzyme-immobilized system, can be used in urea estimation.

EXPERIMENTAL

Materials

Urease (EC 3.5.1.5 from jack beans, Type III), Bradford reagent, and GMA were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). The CFP (no. 393) used for grafting was obtained from Sartorius, Germany. CAN, nitric acid, acetone, and all other analytical grade chemicals were purchased from CDH, India. All the buffers were prepared in milli Q water.

Graft copolymerization

The grafting of GMA onto filter paper was carried out by chemical initiation method using CAN as an initiator. Grafting was done under nitrogen atmosphere in a glass ampoule, fitted with gas inlet. Distilled water was taken in the reaction ampoule, to which a freshly prepared 10-mL solution of CAN ($1.0–6.0 \times 10^{-3}$ molL^{-1}) dissolved in 0.1*M* nitric acid was added followed by 0.5–10% GMA (w/v), and the final volume of the reaction mixture was 40 mL. Preweighed CFP (5 cm × 2 cm in size) was immersed in the grafting mixture and nitrogen was purged into the ampoule to remove air from the grafting solution. The reaction flask was then placed in a temperature-controlled oil bath where the grafting was carried out for the desired time period under constant magnetic stirring. The CFP was washed with acetone taken in a conical flask and placed on mechanical shaker for 16 hrs with change of solvent at 4-hrs interval to remove homopolymer and unreacted monomer from the filter paper. Later on, the GMA-grafted CFP (CFP-g-GMA) was dried and weighed until constant weight was reached. The grafting percentage and grafting efficiency were determined as:

$$\%\text{Grafting } (\%G) = \frac{W_a - W_b}{W_b} \times 100$$

$$\%\text{Grafting Efficiency } (\%GE) = \frac{W_a - W_b}{(W_a - W_b) + W_h} \times 100$$

where, W_b and W_a are weight of the CFP before and after the grafting process, respectively, and W_h is the weight of homopolymer.

From the modified CFP, circular discs of 0.5-cm diameter were punched out using a paper punch.

Characterization

ATR-FTIR spectroscopy

Attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectral analysis of the ungrafted and GMA grafted CFP was recorded with a Perkin–Elmer spectrum one spectrometer.

Thermo Gravimetric Analysis

TA 2100 thermal analyzer having a 951 TG module was used for the thermal characterization of the various grafted CFP. Thermogravimetric analysis of sample was done in the range of 40–400°C in nitrogen atmosphere (flow rate = 60 cm^3/min) at a heating rate of 10°C/min. A sample mass of 10 ± 2 mg was used.

Scanning Electron Microscopy

Scanning electron micrographs of the modified and unmodified CFP were obtained using STEREOSCAN 360 (Cambridge Scientific Industries Ltd., Cambridge, UK) scanning electron microscope, after coating the sample with silver. A thick layer of silver metal is used to provide conduction.

Immobilization of urease

The CFP-g-GMA discs (surface area of 0.39 cm^2 for each disc) were equilibrated in phosphate buffer (PB, 50 m*M*, pH 7.4) for 30 min, and then transferred to urease solution (0.5–10 mg/mL of PB) in microplate wells with one disc in each well.

Immobilization was carried out at 4°C for 16 hrs with occasional shaking. After this period, enzyme immobilized discs were removed from the enzyme solution and washed with the same buffer three times. Discs were then stored at 4°C in fresh buffer until use.

Protein assay

The amount of urease immobilized onto single-grafted CFP disc was determined by measuring the initial and final concentrations of protein within the immobilization medium and was calculated as:

$$Q = \{C_i - C_t\} \times V$$

where Q is the amount of urease immobilized/disc (mg), C_i and C_t are the concentrations of the urease (mg/mL) in the initial solution and in the solution after immobilization, respectively, V the volume of the aqueous phase (mL).

The amount of protein in the enzyme solution was determined using Coomassie Brilliant blue as described by Bradford,[23] at wavelength (λ) = 595 nm.

Activity assay of free and immobilized urease

Free and immobilized urease activities were determined spectrophotometerically at wavelength (λ) = 405 nm. The method is based on the hydrolysis of urea to ammonium.[21]

Free urease assay

To 0.9 mL of assay buffer (0.05*M* Tris-acetate buffer, pH 7.4), 0.1 mL of enzyme solution is added and incubated with 1 mL of urea solution (10–320 mg/dL) at 37°C for 10 min. The enzymatic reaction is stopped by the addition of 1 mL of 10% trichloroacetic acid. An aliquot (1 mL) of the reaction mixture (total 3 mL) is transferred to a 50-mL volumetric flask and 1-mL Nessler's reagent is added with swirling and the volume made upto 50 mL with distilled water. The amount of ammonia produced was determined spectrophotometrically by measuring the intensity of the yellow-colored compound formed after the addition of Nessler's reagent.

Immobilized urease assay

For the determination of immobilized urease activity, two discs were taken together for one reaction and preincubated in assay buffer at 37°C for 2 min and then incubated for 20 min with urea solution (10–320 mg/dL) with intermittent shaking. After the desired time interval, the enzyme immobilized discs were separated from the reaction mixture, washed, and refrigerated (4°C) in buffer for reuse. An aliquot of the reaction mixture was used to develop color with Nessler's reagent as done for the soluble enzyme.

RESULTS AND DISCUSSION

Graft copolymerization

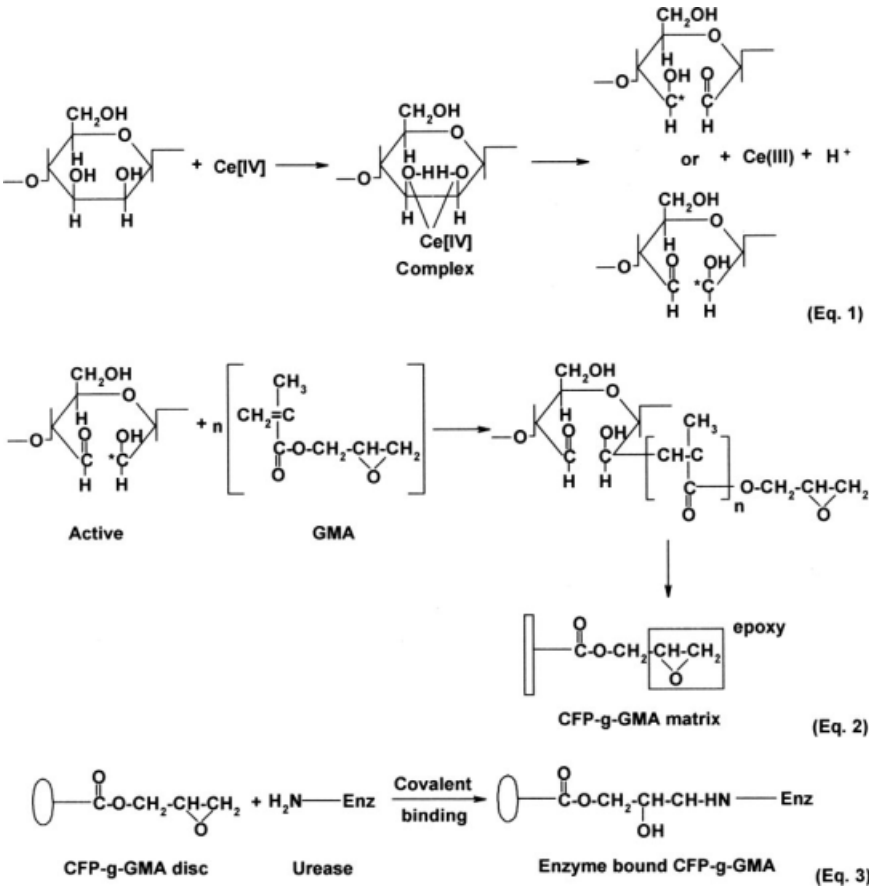
The grafting of GMA onto CFP was carried out by free-radical initiated process. The chemical initiator used, CAN, is soluble in water but the monomer GMA is reported to be soluble in the presence of methanol in the aqueous bath. Although many researchers have used a 50 : 50 methanol : water mixture for grafting,[24–26] the literature reports the maximum graft add-on in purely aqueous medium for grafting of GMA onto cellulose.[7,27] In this work, therefore, a pure aqueous grafting medium was used and the monomer GMA was maintained in an emulsified condition by constant magnetic stirring. Fine globules of GMA were found suspended in the aqueous medium and did not show any tendency to coalesce to form a separate layer during the course of grafting.

The probable reaction mechanism of the present study is presented in Scheme 1. The active centers are directly produced on the cellulosic backbone of the filter paper and no charge-transfer mechanism is necessary to initiate cellulose graft copolymer formation.[28] Cerium(IV) ions in acidic solution form chelates with the hydroxyl groups on carbons C-2 and C-3 of the anhydroglucose unit of CFP forming cellulose–ceric complex. Transfer of electrons from CFP to Ce(IV) gives Ce(III) which dissociates from the chelate. The anhydroglucose ring is scissioned between carbons C-2 and C-3 and a short-living radical is formed [eq. (1), Scheme 1]. In the presence of monomer GMA, grafting reactions are initiated to produce graft copolymer with the glycidyl pendant group [eq. (2), Scheme 1]. Termination of the graft copolymer takes place through the combination of radicals.

To optimize the conditions of grafting, effects of various parameters, such as monomer and initiator concentrations, reaction time, and reaction temperature on the graft level were studied.

Effect of monomer concentration

The influence of the monomer concentration on grafting reaction was studied using varied amounts of GMA ranging between 1 and 10%, taken w/v at different temperatures while other reaction parameters were kept constant. As can be seen from the



Scheme 1 Reaction scheme for grafting of GMA on CFP and the immobilization of urease onto the grafted surface. Equation 1. Activation of cellulose filter paper surface. Equation 2. Grafting of GMA. Equation 3. Immobilization of urease.

results, the grafting percentage increased with increasing monomer concentration from 1 to 5% (Fig. 1). However, beyond this value, the monomer concentration had a small effect on the percent grafting and became a decreasing tendency. A similar trend was observed at all the temperature range studied (40 to 70°C). The GMA concentration of 5% was therefore recognized as an optimum monomer concentration for grafting reaction. The enhancement in the rate of grafting on increasing the monomer concentration to an optimum value could be ascribed to the greater availability of monomer molecules to the free radicals (grafting sites) on the CFP. The slight decreasing trend in percent grafting at higher monomer concentration may be due to the number of free-radical sites available on the surface becoming a limiting factor and higher affinity of the monomer for its homopolymer over surface radicals.

Effect of initiator concentration

The effect of the initiator concentration was studied at various temperatures by keeping the concentration of GMA at 5% and the polymerization time as 25 min. The result obtained by changing the initiator concentration from 1×10^{-3} to 6×10^{-3} molL^{-1} is shown in Figure 2. The percent grafting reached a maximum value at the critical CAN concentration of

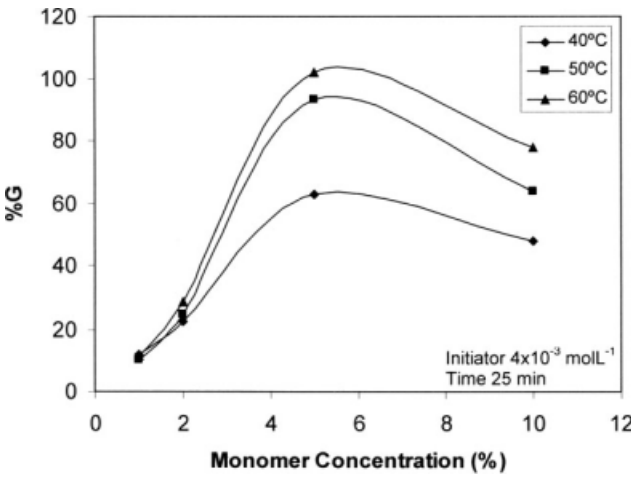


Figure 1 Effect of monomer concentration on the grafting reaction.

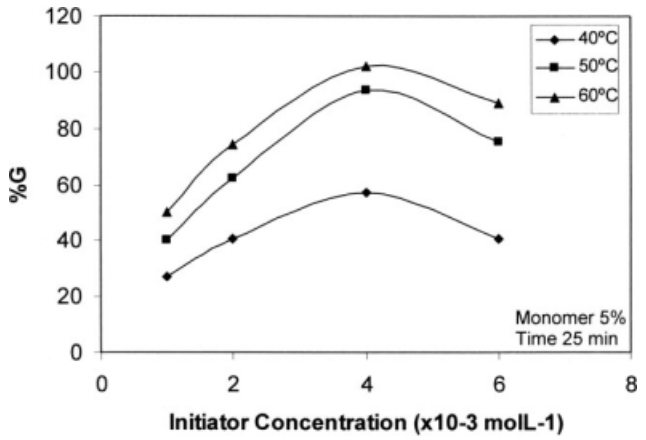


Figure 2 Effect of initiator concentration on the grafting reaction.

4×10^{-3} molL^{-1} for all the temperatures studied. Any further increase in CAN concentration was accompanied by a slight decrease in grafting yield. Increasing the initiator concentration initially increases the concentration of free radicals—formed through decomposition of the initiator—actively involved in many graft polymerizations within the polymerization medium. These radicals can directly interact with the surface to form active sites and may also initiate less homopolymer formation. A further increase in the amount of CAN over 4×10^{-3} molL^{-1} results in the enormous primary radicals (surface radicals) and growing macroradicals of the side chains, which may interact with each other, resulting in a termination of reactive sites and hence a reduction in the percent grafting.

Effect of reaction time

The influence of the polymerization time on the percent grafting is shown in Figure 3. With an increase in the polymerization time, the graft level increased initially and reached a plateau in 25 min, which was leveled off with further increase in the polymerization period. When the reaction time is increased, more time is available to the free radicals for reaction and it therefore results in higher percent grafting. After sometime, all the initiator and monomer molecules are consumed and hence there is no further change in the percent grafting observed. Thus, the optimum polymerization time recorded was 25 min.

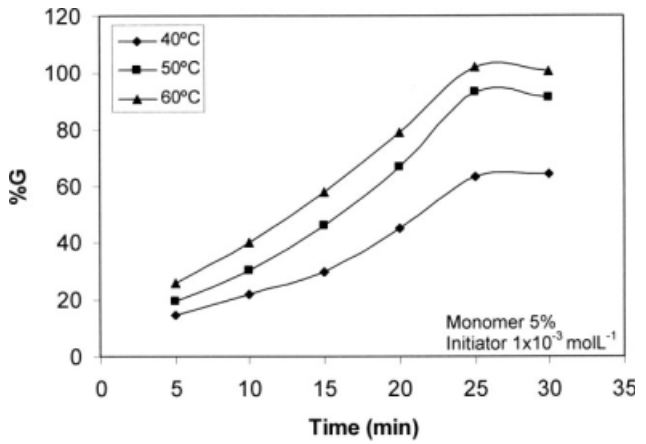


Figure 3 Effect of polymerization time on the grafting reaction.

Effect of reaction temperature

Determining an optimum temperature is one of the significant factors to standardize the grafting reaction. To study the effect of polymerization temperature on the reaction parameters, the grafting of GMA onto the CFP was carried out at different temperatures ranging from 40 to 70°C. The percent grafting increased by increasing the polymerization temperature from 40 to 60°C and then slightly decreased with further increase of temperature to 70°C (Fig. 4). This can be attributed to the fact that with an increase in temperature, decomposition rate of the initiator increases. Therefore, the amount as well as the mobility of free radical increases, which results in a higher level of grafting. However, further increase in reaction temperature beyond 60°C leads to mutual termination of various free radicals and various hydrogen abstraction and chain transfer reactions leading to decrease of percent grafting. The grafting efficiency of GMA onto CFP shows a decreasing pattern with an increase in temperature from 40 to 70°C.

Characterization

ATR-FTIR spectroscopy

FTIR spectra of ungrafted and grafted filter paper samples are presented in Figure 5. FTIR spectrum of CFP exhibits characteristic absorption bands at 3400

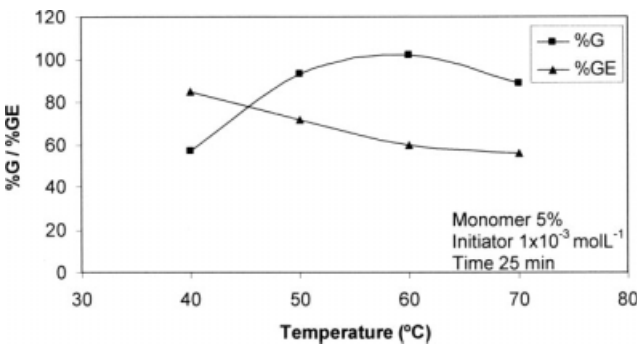


Figure 4 Effect of polymerization temperature on percent of grafting (%G) and grafting efficiency (%GE).

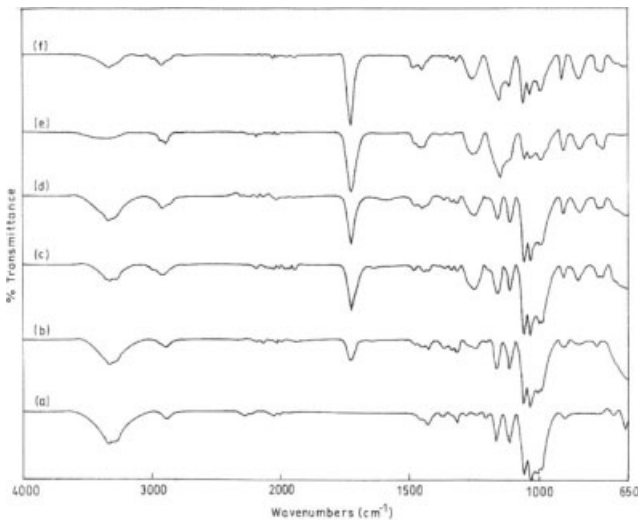


Figure 5 FTIR spectra of (a) ungrafted CFP and (b–f) GMA-grafted CFP with varying percent grafting [(b) 20%; (c) 40%; (d) 60%; (e) 80%; (f) 100%].

and 2920 cm^{-1} because of O—H stretching and C—H stretching vibrations of cellulose. Other characteristic absorption bands of CFP appear at 1426 and 1023 cm^{-1} because of C—H bending and O—H bending vibrations, respectively [Fig. 5(a)]. The grafted surface exhibits new functional groups apart from the characteristic absorptions of CFP surface [Fig. 5(b–d)]. The observed additional peaks at 906 and 845 cm^{-1} are assigned to epoxide groups of grafted GMA. Another peak observed at 1730 cm^{-1} corresponds to the carbonyl functional group of the grafted GMA. These results confirmed the grafting of GMA onto the filter paper surface. Additionally, the intensity of these characteristic peaks, corresponding to those of GMA, increased with an increase in the graft level. GMA grafting on CFP was further confirmed by the weak band intensity at 3400 and 1023 cm^{-1} of CFP-g-GMA spectra as compared with the spectrum of the plain cellulose FP. Weakening of the band is due to the utilization of some —OH groups of cellulose during the formation of the graft copolymer as shown in Scheme 1. Also, the complete absence of characteristic absorbance band of GMA double bond at 1637 cm^{-1} in CFP-g-GMA spectra confirms the opening of this bond during polymerization reaction and hence confirming the grafting.

Thermo Gravimetric Analysis

Thermo gravimetric analysis thermograms of ungrafted and grafted CFP samples were studied to understand their thermal behavior (Fig. 6). The ungrafted CFP show two stages of weight loss: the first stage, below 100°C, is associated with dehydration of the sample and the second stage is due to the decomposition of the cellulose matrix (235 to 475°C). The TG curve of PGMA show one stage degradation due to random chain scission, which begins at around 160°C and reaches maximum at 280°C. Similar observation has also been reported by Caykara and Alaslan.[29] The GMA grafted CFP samples, on the other hand, follow three-step degradation patterns. The initial loss due to dehydration is followed by second stage loss from 168°C to 274°C which may be related to the degradation of poly(GMA) chains. The subsequent loss of CFP matrix starts thereafter. The second stage related to the degradation of GMA grafted surface show an increase in weight loss with an increase in grafting level. This is expected because the poly(GMA) component also increases along with an increase in percent grafting.

Scanning Electron Microscopy

The electron micrographs corresponding to ungrafted and grafted CFP are depicted in Figure 7 (a–d). Before grafting the fibers in CFP has a smooth and relatively homogeneous appearance. Grafting of GMA developed roughness and introduced heterogeneity along and across the fibers which increased with the increase in degree of grafting. This may be the outcome of incompatibility of the hydrophobic

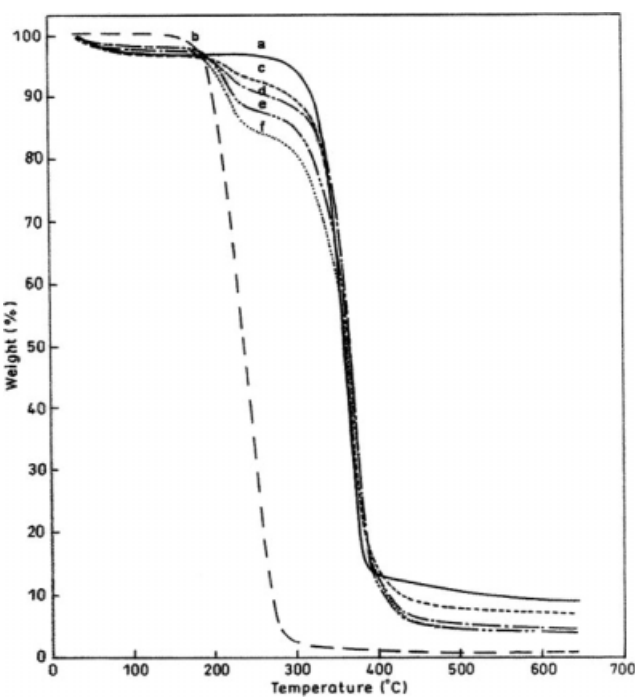


Figure 6 TGA themograms of (a) ungrafted CFP, (b) homopolymer PGMA, and (c–f) GMA-grafted CFP with varying percent grafting [(c) 20%; (d) 50%; (e) 70%; (f) 100%].

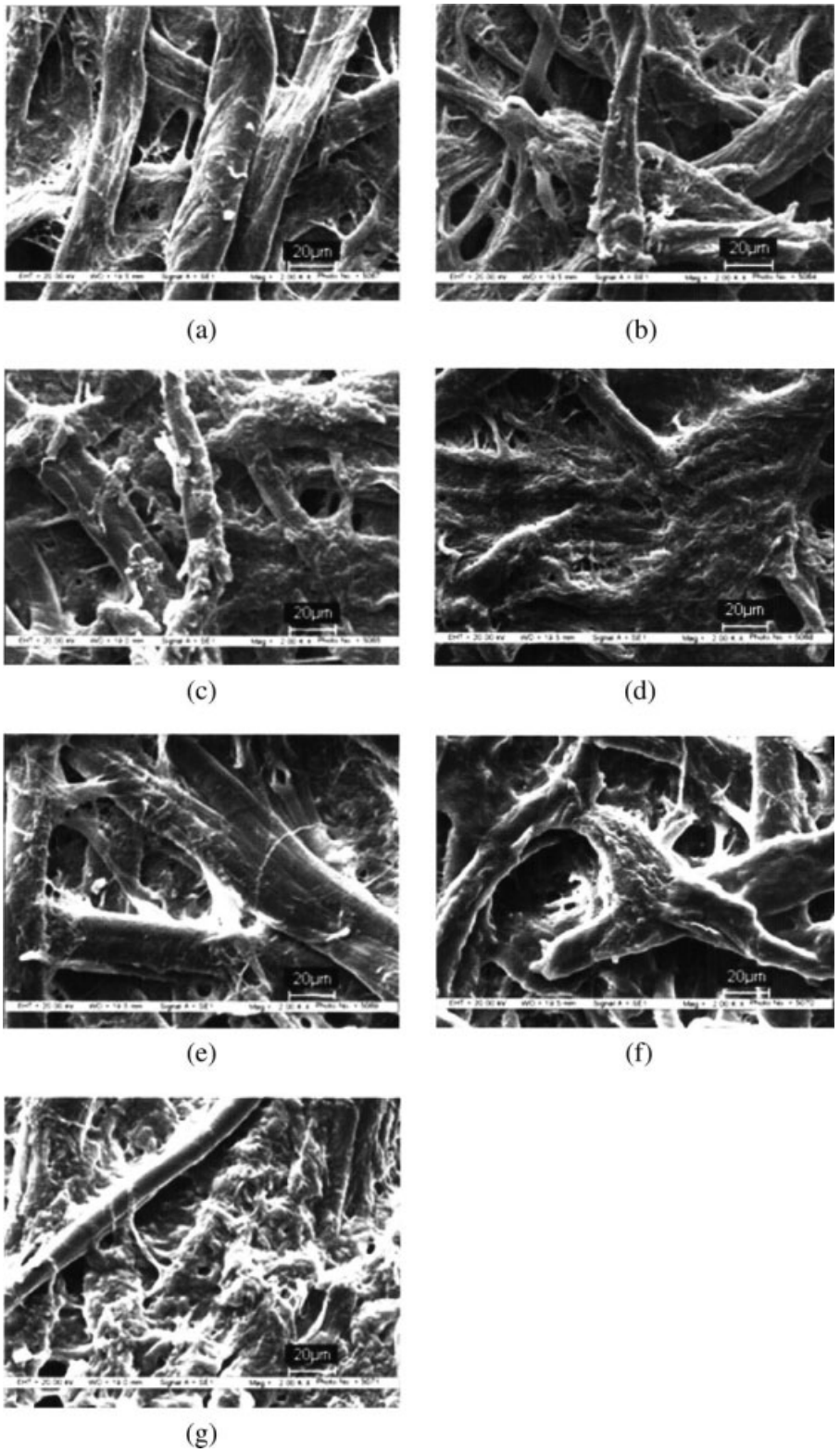


Figure 7 Scanning electron micrographs of (a) ungrafted CFP, (b–d) GMA-grafted CFP with varying percent grafting [(b) 20%; (c) 70%; (d) 100%] and (e–g) GMA-grafted CFP after urease immobilization [(e) 20%; (f) 70%; (g) 100%].

poly(GMA) grafts with the hydrophilic cellulosic filter paper matrix. After the immobilization of urease, again there are changes in the surface topography of modified CFP, which becomes smooth thereby proving the immobilization of enzyme [Fig. 7(e–g)].

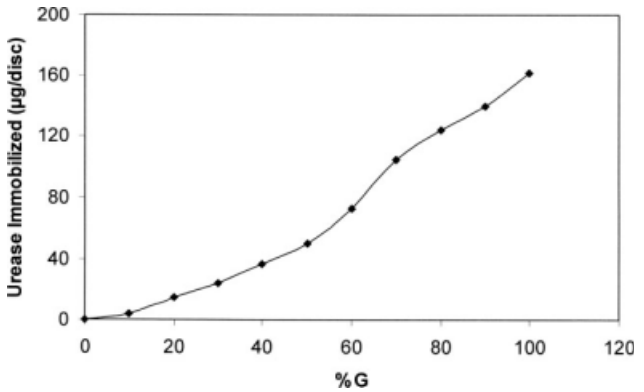


Figure 8 Effect of grafting percentage on enzyme (urease) loading (µg/disc).

Immobilization of urease

The GMA grafted CFP discs have been effectively used for immobilization of urease. CFP was chosen as the carrier because of its biodegradable nature, low price, easy availability, and ecofriendliness, and the functionality was introduced on its surface for covalent coupling of biomolecules. To impart functionality to the surface, GMA, which has reactive epoxide pendant, was grafted onto the filter paper through double bond present in the acrylic backbone of the monomer. Immobilization of urease onto CFP-g-GMA discs was achieved by covalent coupling of epoxy groups of the support with amino groups of the enzyme [eq. (3), Scheme 1). The CFP-g-GMA discs with varied grafting percentage (from 10 to 100%) were studied for effective enzyme immobilization and the amount of enzyme coupled to the surface with various percent grafting is presented in Figure 8. The results showed an increase in the amount of enzyme bound, from 4- to 162-µg urease/disc, with an increase in GMA content of the disc. As can be seen in the FTIR spectra of CFP-g-GMA discs, their is a gradual upgradation in the intensity of epoxy peak at 906, which exhibits a higher epoxy content available for enzyme immobilization on the discs with higher graft level. Also, with an increase in concentration of urease in the immobilization medium (from 0.5 to 10 mg/ml) there was an increase in the immobilization efficiency which leveled off at an enzyme concentration of 5 mg/ml (Fig. 9). This may be attributed to the competitive binding (enzyme–enzyme interaction) of enzyme molecule through active epoxy groups and stearic hindrance at higher enzyme concentration. Although the amount of enzyme bound to the support increased after 5-mg/mL enzyme concentration, the activity did not increased accordingly, denoting the constant number of active sites for enzyme on the surface of the disc. The urease concentration of 5 mg/mL was, therefore, used for enzyme immobilization on the discs, which are to be used for further experiments.

Urease activity

The hydrolytic activity of immobilized urease disc with varied enzyme loading as per different percent grafting was examined at a constant substrate (urea) concentration of 320 mg/dL. As seen in the Figure 10, the activity of the urease immobilized on discs with 70% graft level (total 210 µg for two discs) was maximum and significantly higher than that of enzyme immobilized disc with 100% graft level (total 324 µg for two discs), which although exhibited maximum enzyme loading. The figure shows a gradual increase in the activity of immobilized enzyme on discs with 10% graft level to 70%, after which a fall in the activity is observed. This lower activity recorded with higher enzyme load is probably due to local aggregation of enzyme presenting sterric hindrance and/or difficulty in diffusion of substrate molecule to the active sites of the enzyme bound to the surface. Therefore, 70% GMA grafted urease immobilized discs were considered for further experiments.

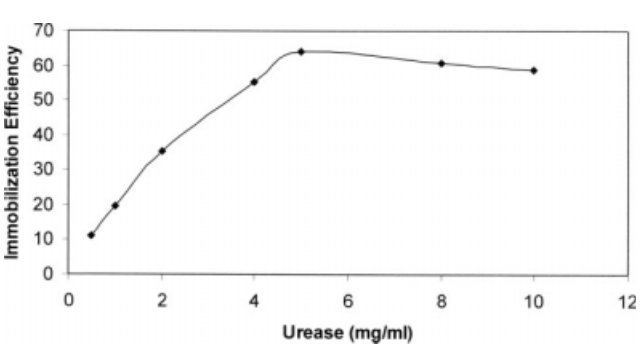


Figure 9 Effect of enzyme concentration on the immobilization of urease on CFP-g-GMA discs (70% graft level).

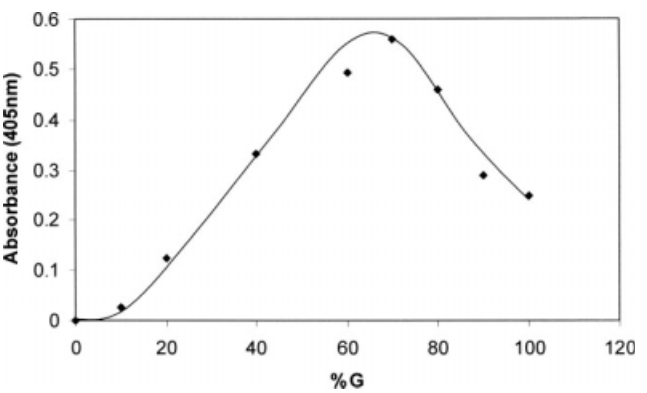


Figure 10 Activity of enzyme immobilized on CFP-g-GMA discs with various graft levels.

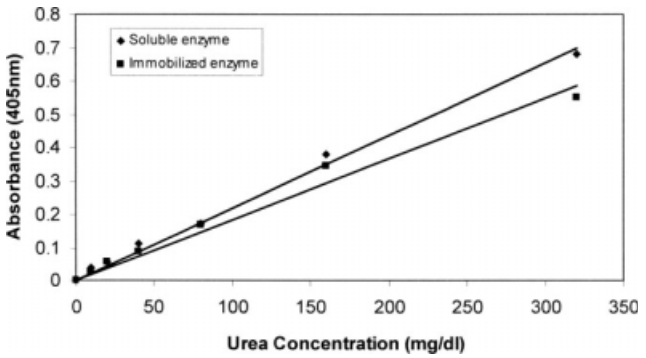


Figure 11 Activity of soluble and immobilized enzyme (on CFP-g-GMA (70%) discs) at different urea concentration using standard substrate (urea) solutions.

The hydrolytic activity of the free and immobilized urease preparations was compared at different substrate (urea) concentrations. Although, with an increase in substrate concentration, a linear increase in the activity was observed for both the preparations, but, a fall in the immobilized enzyme activity was recorded at all substrate concentrations when compared with the soluble enzyme (Fig. 11). This fall in the activity is due to lower affinity of the immobilized enzyme for its substrate which has resulted due to the structural changes in the enzyme induced by the interaction of the macromolecule with the support. Alternatively, the increased diffusional resistance encountered by the substrate in its approach to the catalytic site could be the reason for the decline in immobilized enzyme activity. Similar results have been reported for urease immobilization on butyl methacrylate grafted nylon membranes and poly(2-hydroxyethyl methacrylate-comethacryloyl-L-histidinemethylester) microspheres and other surfaces.[30,31]

The durability of the immobilized enzyme after repeated cycles of use is also very important. Urease immobilized CFP discs reusability was investigated and the activity retention of the immobilized enzyme after repeated hydrolysis reaction was studied, the discs being washed in buffer after every subsequent usage. The immobilized enzyme urease retained all of its activity until four consecutive uses (at an interval of 24 hrs) and lost 15–20% of its original activity in fifth cycle of reuse. This ensured better immobilization of the enzyme on CFP disc by covalent coupling through grafted GMA. A higher stability of the immobilized enzymes has been reported by several investigators as a result of prevention of autodigestion and thermal denaturation due to the fixation of enzyme molecules on the matrix.[32,33] This increased stability, reusability, and handling ease of the enzyme immobilized discs make it possible for the utilization of this enzyme in the degradation of urea for various purposes such as, biosensors, diagnostics for urea assay, for dialysate treatment in kidney dialysis, treatment of contaminated water, etc.

CONCLUSION

In this study, GMA was successfully grafted onto CFP under an inert atmosphere, using CAN as an initiator. Degree of grafting varied from 10 to 102% under different reaction conditions and a maximum of 102% was recorded at 60°C in 25 min with 4×10^{-3} molL^{-1} of initiator (CAN) concentration and 5% GMA monomer. The enzyme urease was covalently immobilized onto the modified-CFP surface via epoxy groups of grafted GMA, which showed an increased enzyme immobilization with an increase in the GMA content of the grafted CFP, the maximum being 162-μg urease/disc at highest percent grafting (102%). Although the highest immobilization efficiency was attained at 100% graft level, the enzyme immobilized on discs with 70% graft level (105-μg urease/disc) gave the highest enzymatic activity. It can be concluded that the CFP surface with 70% GMA grafting showed optimum surface epoxy groups required for efficient urease immobilization and such enzyme immobilized discs could be used effectively with good reusability in urea detection, estimation, and degradation.

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