Synthesis and Characterization of Binary Copolymers of Methyl Methacrylate with Glycidyl Methacrylate and 2-Hydroxy Ethyl Methacrylate as Carriers for Cellulase

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ABSTRACT: Cellulase was immobilized directly on methyl methacrylate-glycidyl methacrylate copolymer (MMA-*co*-GMA) and methyl methacrylate-2-hydroxy ethyl methacrylate copolymer (MMA-*co*-HEMA) by covalent attachment and crosslinking methods. The properties of the immobilized cellulase were investigated and compared with those of the free one. For the assays carried out through crosslinking method at 25°C and pH 7, the retained activities were found to be 91.92% and 74.63%, respectively, for MMA-*co*-GMA and MMA-*co*-HEMA crosslinked with 0.1% of 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT), respectively. The immobilized cellulase had better stability and higher retained activities with respect to pH, temperature, and storage stability than the free one. In the repeated use experiments, the immobilized cellulase using (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%) did not change after 10 and eight times of repeated use and maintained 67% and 62% from their original activities after 25 times, respectively. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 629–638, 2010

Key words: immobilized cellulase; MMA-*co*-GMA; MMA-*co*-HEMA; covalent attachment; crosslinking

INTRODUCTION

Synthetic polymers are attractive carriers for enzymes immobilization because they are resistant to deterioration by microorganism and chemicals in acidic and basic environments.[1,2] They have an additional advantage of being relatively easy to modify, and therefore, their properties can be changed to adjust them to a particular enzyme and process requirements.[3,4] There are many methods, such as adsorption, crosslinking, covalent binding, etc., for enzyme immobilization.[5,6] The methods and supports employed for enzyme immobilization are chosen to ensure the highest retention of enzyme activity and its stability and durability.[7–10]

Cellulases are enzymes that hydrolyze the β-(1–4) linkages in cellulose. They are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and cellobiase (β-glucosidase, EC 3.2.1.21). Cellulases are commonly used in various industries, including the food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper, as well as in research development.[11,12] Free cellulase has a low stability, and we cannot recycle it for further use. Immobilization of cellulase may be one of the better ways to increase its utility.[13]

This study outlines the preparation of methyl methacrylate-glycidyl methacrylate and methyl methacrylate-2-hydroxy ethyl methacrylate copolymers (MMA-*co*-GMA) and (MMA-*co*-HEMA) prepared by emulsion polymerization. The matrix is a copolymer and is neither as rigid as pure poly (methyl methacrylate) nor as hydrophilic as pure poly(2-hydroxyethyl methacrylate). The immobilization of cellulose was carried out through covalent attachement and crosslinking methods. The hydroxyl groups of the hydroxyl ethylmethacrylate were either reacted directly with the residual carboxylic groups in α-amylase enzyme (covalent attachement method) or crosslinked with 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT) as a crosslinking agent and then were reacted with the cellulase (crosslinking method).

EXPERIMENTAL

Materials

Cellulase (EC 4.2.1.4 from *Aspergillus niger* with activity 108 U/g) ethylene glycol dimethacrylate

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(EGDMA), glycidyl methacrylate (GMA), Folin reagent (2N), sodium dihydrogen phosphate anhydrous, and sodium dodycyl benzyne sulfonate (SDBS) were purchased from Fluka; methyl methacrylate (MMA) and starch were obtained from Merck, 2-hydroxyethyl methacrylate (HEMA) was obtained from Sigma–Aldrich; bovine serum albumin from was purchased Aldrich; CMCT was purchased from Sigma; sodium phosphate dibasic anhydrous was purchased from El Gomhouria Co., Egypt; glucose and maltose was obtained from BDH; and 3,5-dinitrosalicylic acid (DNSA) was obtained from Panreac. All monomers were distilled by passing through alumina basic column and then stored at 4°C until use.

Methods and Techniques

Methods

Preparation of MMA-co-GMA and MMA-co-HEMA. Copolymers of MMA-*co*-GMA and MMA-*co*-HEMA were prepared by the emulsion polymerization technique. The reactions were carried out in a 250-mL three-necked flask fitted with a condenser and a thermometer. The system also had a nitrogen inlet and was stirred with a magnetic stirrer. Distilled water (100 mL), SDBS (0.5 g), potassium persulphate (KPS)/ glucose (0.27/0.18 g), as a novel safe redox pair system, were added into the reaction vessel and heated to 70°C while flushing nitrogen through the solution. Then MMA (6.93 mL) and GMA (4.09 mL) [in (MMA-*co*-GMA) preparation] and MMA (6.93 mL) and HEMA (3.63 mL) [in (MMA-*co*-HEMA) preparation] with/ without EGDMA (0.198 or 0.99 mL) as a crosslinker were added. The reaction ingredients were stirred vigorously for 4 h at 70°C. The prepared stable emulsion copolymer was precipitated by sodium chloride solution and separated by filtration, washed several times with distilled water, methanol, and hot distilled water, dried in an electric oven at 50°C and weighed.

Support crosslinked with CMCT. MMA-*co*-GMA and MMA-*co*-HEMA (0.1 g) were added to a CMCT solution (1 mg in 1 mL phosphate buffer, pH 7), kept in a shaking water bath for 4 h at 25°C and left at that temperature overnight. The activated copolymers were separated and washed three times with phosphate buffer (5 mL).

Immobilization of cellulase. ***Covalent attachment method.*** MMA-*co*-GMA and MMA-*co*-HEMA (0.1 g) was added to the cellulase solution with different concentrations [(1, 2, 3, 4, and 5) mg in (1 mL, 0.02 M) phosphate buffer pH 7], and the immobilization reaction was carried out for 24 h at 25°C in a shaking water bath. The precipitates were filtered, and the unbound cellulase was removed by washing three times with (5 mL) phosphate buffer. The immobilized α-amylase polymer samples were stored at 4°C until use.

Crosslinking method. The previous crosslinked MMA-*co*-GMA and MMA-*co*-HEMA with CMCT (0.1 g) was added to the cellulase solution with different concentrations [(1, 2, 3, 4, and 5) mg in (1 mL, 0.02 M) phosphate buffer, pH 7], and the immobilization reaction was carried out for 4 h at 25°C in a shaking water bath. The precipitates were filtered, and the unbound cellulase was removed by washing three times with (5 mL) phosphate buffer. The immobilized cellulase polymer samples were stored at 4°C until use.

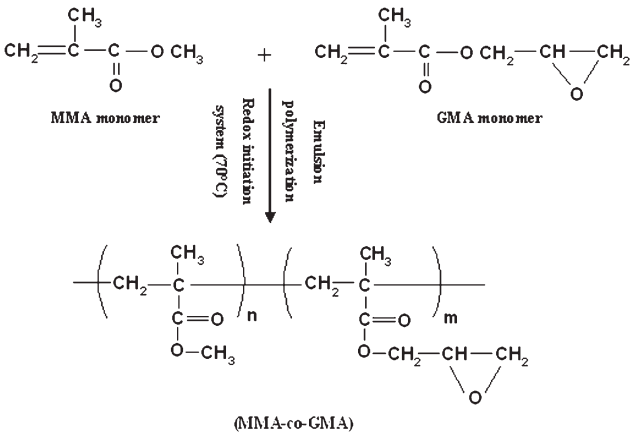
Determination of the amount of immobilized cellulase. The Lowry method[14] was used to determine the cellulase content in solution. After the immobilization process, the supernatant and the washing solutions were collected. The cellulase concentration was determined by comparing with the standard curve constructed using bovine serum albumin with known concentrations. The amount of immobilized cellulase was determined from the initial cellulase amount present in the cellulase coupling solution substracting the final total cellulase amount present in the remaining coupling solution.[15] The coupling yield (%) of the cellulase was then calculated from the amount of cellulase coupled on the polymeric carriers by the initial total amount of the cellulase present in the coupling solution according to the following equation:

$$\text{Enzyme coupling yield \%} = \frac{\text{amount of cellulase coupled}}{\text{amount of cellulase introduced}} \times 100$$

Assay of cellulase activity. The activity of cellulase was measured by two methods:

1. Activities of free and immobilized cellulase were determined by incubating the enzyme for 3 min with 0.5% CMC in phosphate buffer (0.02 M, pH 7.0) at 25°C using DNSA reagent as color developing agent.[16] The reducing sugar produced was measured spectrophotometrically at 540 nm [using UV/visible recording spectrophotometer (Shimadzu UV-2401 PC double-beam)] with glucose as a standard.[17,18] One unit (U) of activity was defined as the amount of enzyme that produces one micromole of glucose equivalent per minute under above-mentioned conditions. The specific activity of free and immobilized cellulase was calculated according to the following equation:

$$\text{Glucose concentration(mg/dL)} = \frac{\text{unknown absorbance}}{\text{standard absorbance}} \times 100$$



Scheme 1 The preparation of MMA-*co*-GMA through emulsion polymerization.

The retained activities yield of the immobilized cellulase on the different polymeric supports were calculated according to the following equation:

$$\text{Retained activity yield}(\%) = \frac{\text{specific activity of immobilized cellulase}}{\text{specific activity of free cellulase}} \times 100$$

2. Activities of free and immobilized cellulase were determined by incubating 1 mL enzyme buffer indicator solution with 10 μL of sample solution for 10 min in waterbath at 37°C. The reducing sugar produced was measured spectrophotometrically at 500 nm with glucose kit standard. The specific activity of free and immobilized cellulase was calculated according to the following equation:

$$\text{Glucose concentration}(\text{mg/dL}) = \frac{\text{unknown absorbance}}{\text{standard absorance}} \times 100$$

Conversions: 1mg/dL glucose equals 55.5 μM, 0.001% or 10 ppm.

The retained activities yield of the immobilized cellulase on the different polymeric supports were calculated according to the following equation:

$$\text{Retained activity yield}(\%) = \frac{\text{specific acitivity of immobilized cellulase}}{\textit{specific activity of free cellulase}} \times 100$$

All activity measurement experiments were carried out at least twice (generally three times), and the relative standard deviations were found to be less than 1%.

Parameters affecting cellulase activity

1. The effect of pH on cellulase activity was investigated in the range 3–9 for both free and immobilized one.
2. The effect of temperature on cellulase activity was investigated in the range of 30°C–100°C for both free and immobilized one.
3. Storage stability of free and immobilized cellulase was carried to determine the stabilities of free and immobilized cellulase after storage in phosphate buffer (0.02 M, pH 7) at 4°C for 90 days. The residual activities were then determined as described earlier, and the activity of each preparation was expressed as a percentage of its residual activity compared to its initial activity.
4. To evaluate the reusability of the immobilized cellulase, the matrices were washed with water and buffer after use and then suspended again in a fresh reaction mixture to measure the enzymatic activity.

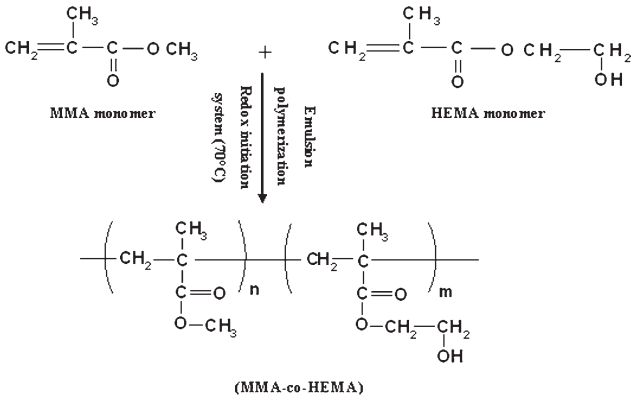
Instruments

FTIR was measured using FTIR spectrometer (Nicolet 670, range from 400 to 4000^{-1}, USA), TGA was measured using thermogravimetric analyzer (7 series Perken Elmar, USA), the shapes of the particles were scanned using scanning electron microscope (SEM) (JXA-840A Electron probe microanalyzer, Jeol), and UV/visible spectrum were measured using Shimadzu UV-2401 PC double-beam spectrometer.

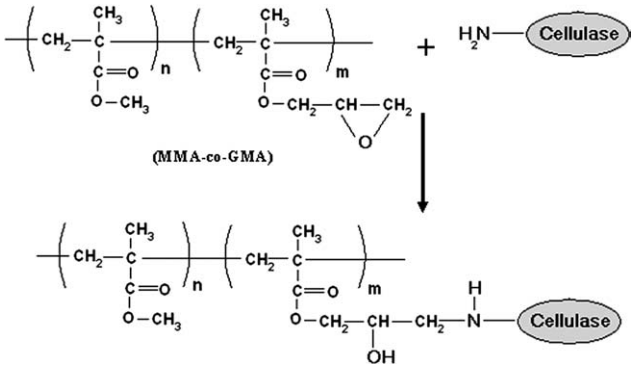
RESULTS AND DISCUSSION

Preparation of MMA-*co*-GMA and MMA-*co*-HEMA matrices

In this study, the emulsion process was chosen as a method of polymerization because of its high



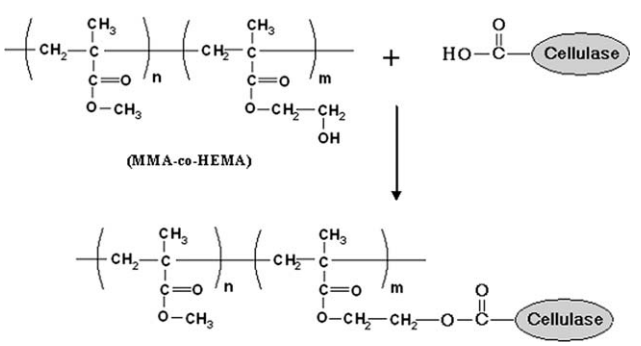
Scheme 2 The preparation of MMA-*co*-HEMA through emulsion polymerization.



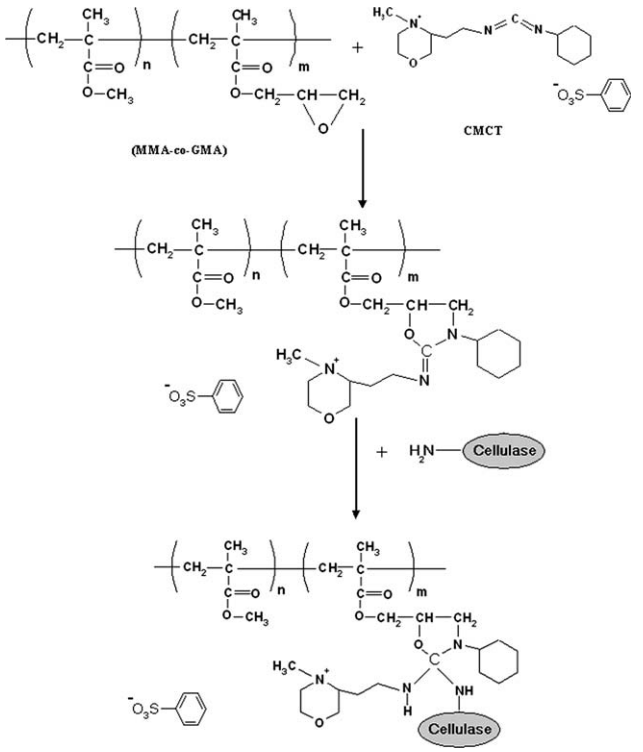
Scheme 3 The possible reaction mechanism between MMA-*co*-GMA and cellulase through covalent attachment method.

conversion (close to 100%), high degree of selectivity, environmental benefits due to the use of an aqueous medium and its usefulness in industry. MMA-*co*-GMA and MMA-*co*-HEMA matrices were prepared as support materials by emulsion polymerization as shown in Scheme (1 and 2). The emulsion process was carried out using an ecofriendly redox system KPS/glucose and SDBS (as an emulsifier) in the presence and absence of EGDMA as a crosslinker. The prepared stable emulsion of copolymers was precipitated by sodium chloride solution and separated by filtration, washed several times with methanol and hot distilled water, dried in an electric oven at 50°C, and weighed. It was found that the polymerization conversion was ~ 99%.

The most frequently used supports in biotechnology for covalent enzyme immobilization have been obtained after activation of synthetic polymers such as poly vinyl alcohol[19] and acrylic polymers.[20] The present method was effective in that the reactive epoxy group could be reacting directly with the amino residuals in the cellulase in only one step without any modification. The —C—N<bonds formed by the epoxide groups are extremely stable, so that the epoxide-containing support matrices could be used for the immobilization of cellulase. This reaction is

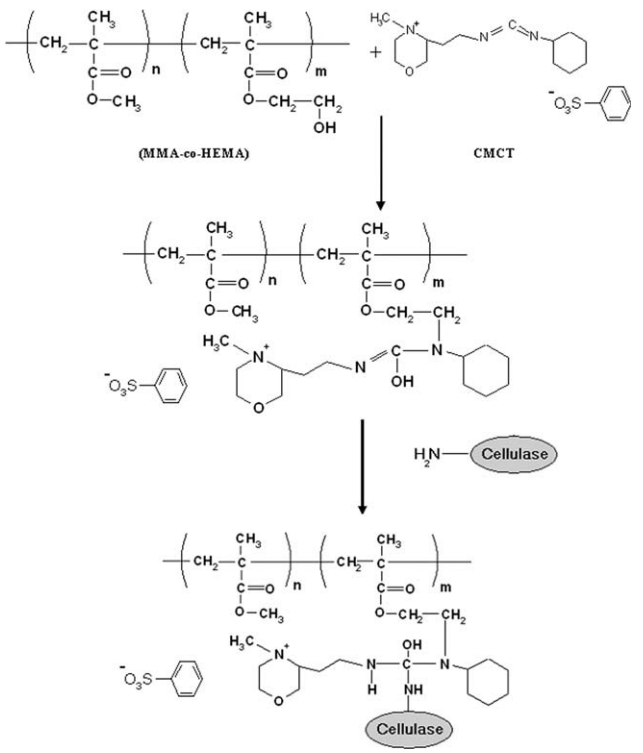


Scheme 4 The possible reaction mechanism between MMA-*co*-HEMA and cellulase through covalent attachment method.



Scheme 5 The possible crosslinking mechanism between the MMA-*co*-GMA support, CMCT (0.1%), and cellulase.

similar to that was carried out by Martin and Anders[21] in trypsin immobilization. Scheme (3) shows the possible covalent immobilization



Scheme 6 The possible crosslinking mechanism between the MMA-*co*-HEMA support, CMCT (0.1%), and cellulase.

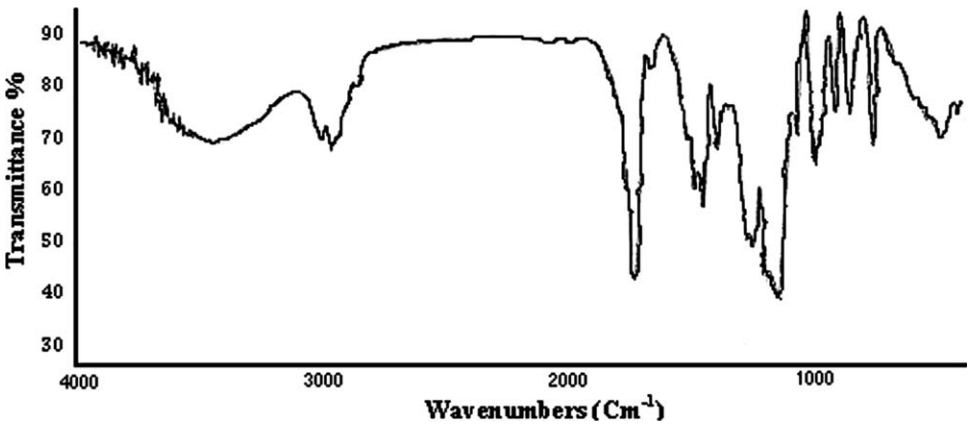


Figure 1 FTIR spectrum of MMA-*co*-GMA.

mechanism between the MMA-*co*-GMA support and cellulase.

The hydroxyl groups of the MMA-*co*-HEMA matrix are very reactive to form a covalent ester bonds with the carboxyl residuals in cellulase. This reaction is similar to that was carried out by Arica et al.[22] but in case of lipase. Scheme (4) shows the possible covalent attachment mechanism between the MMA-*co*-HEMA support and cellulase.

On the other hand, the epoxy groups-containing MMA-*co*-GMA could be reacted with CMCT as a crosslinking agent, and then cellulase was bound to it. The literature is scanty concerning the reaction between the epoxy group and CMCT. Scheme (5) shows the possible crosslinking mechanism between the MMA-*co*-GMA support, CMCT (0.1%) and cellulase. The hydroxyl groups containing MMA-*co*-HEMA could be reacted with CMCT as a crosslinking agent, and then cellulase was bounded to it (crosslinking method). Scheme (6) shows the possible crosslinking mechanism between the MMA-*co*-HEMA support, CMCT (0.1%), and cellulase.

Characterization of MMA-*co*-GMA and MMA-*co*-HEMA

FTIR studies

The FTIR spectrum of MMA-*co*-GMA, as in Figure 1, have the characteristic stretching vibration band of hydrogen-bounded alcohol at 3437 cm^{-1}. Among the characteristic vibrations of both MMA and GMA is the methylene vibration at 2953 cm^{-1}. The vibration at 1730 cm^{-1} represents the ester configuration of both MMA and GMA. The epoxide group gives the band at 845 cm^{-1} and 909 cm^{-1} (epoxy ring vibrations). The FTIR spectrum of MMA-*co*-HEMA, as in Figure 2, show the following characteristic signals: 3525 cm^{-1} (—OH stretching vibration), 3100 cm^{-1}–2900 cm^{-1} (=C—H stretching vibration), 1731.49 cm^{-1} (ester group, C=O stretching vibration), 1320 cm^{-1}–1250 cm^{-1} and 1200 cm^{-1}–1152 cm^{-1} (C—O—C stretching and 1077.14 cm^{-1} (—C—O of C—OH stretching vibrations). The epoxide group gives the band at 845 cm^{-1} and 909 cm^{-1}.

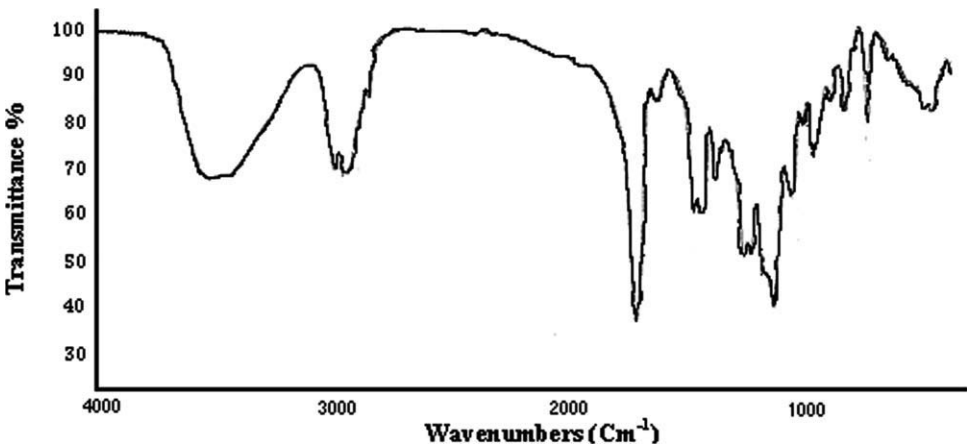


Figure 2 FTIR spectrum of MMA-*co*-HEMA.

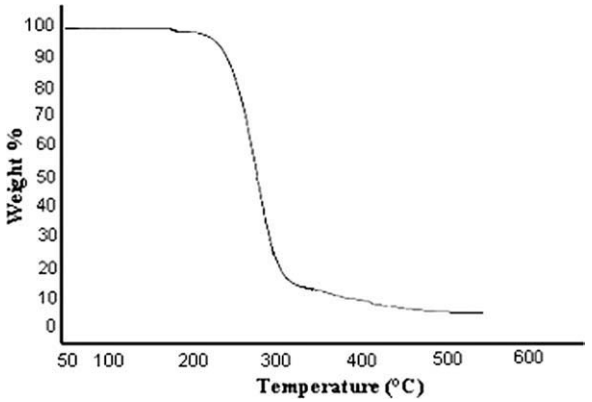


Figure 3 TGA of MMA-*co*-GMA.

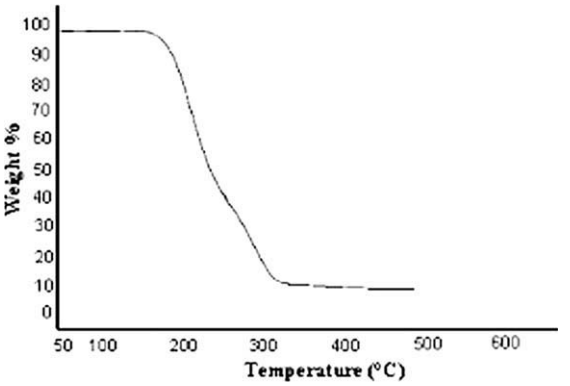


Figure 4 TGA of MMA-*co*-HEMA.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) has been measured for copolymers. It is illustrated from Figures 3 and 4 that small loss in the weight of the samples occurs (1.4% and 1.431%, respectively) from 50°C to 206.7°C and 150°C for MMA-*co*-GMA and MMA-*co*-HEMA respectively. After these degrees, sharp degradation takes place till 337.29°C and 343.33°C (weight loss were 84.576% and 61.519 %), then gradual degradation takes place till 604.6°C and 500°C (weight loss were 9.305% and 31.002%) for MMA-*co*-GMA and MMA-*co*-HEMA respectively, after these degrees complete decomposition occurs.

Scanning electron microscopy

SEM micrographs of MMA-*co*-GMA and MMA-*co*-HEMA show the morphology of these copolymers before and after cellulase immobilization. It is observed from Photos 1 (a,c) that both of MMA-*co*-GMA and MMA-*co*-HEMA have a pours surface before the immobilization process and they have a fibrous surface after cellulase immobilization as shown in Photos 1 (b,d). These observations indicate that the cellulase successfully bound to MMA-*co*-GMA and MMA-*co*-HEMA.

Cellulase immobilization

The immobilization of enzymes onto insoluble polymeric supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports. These immobilized products were intended for use in the construction of artificial organs, biosensors, or bioreactors.[23] The

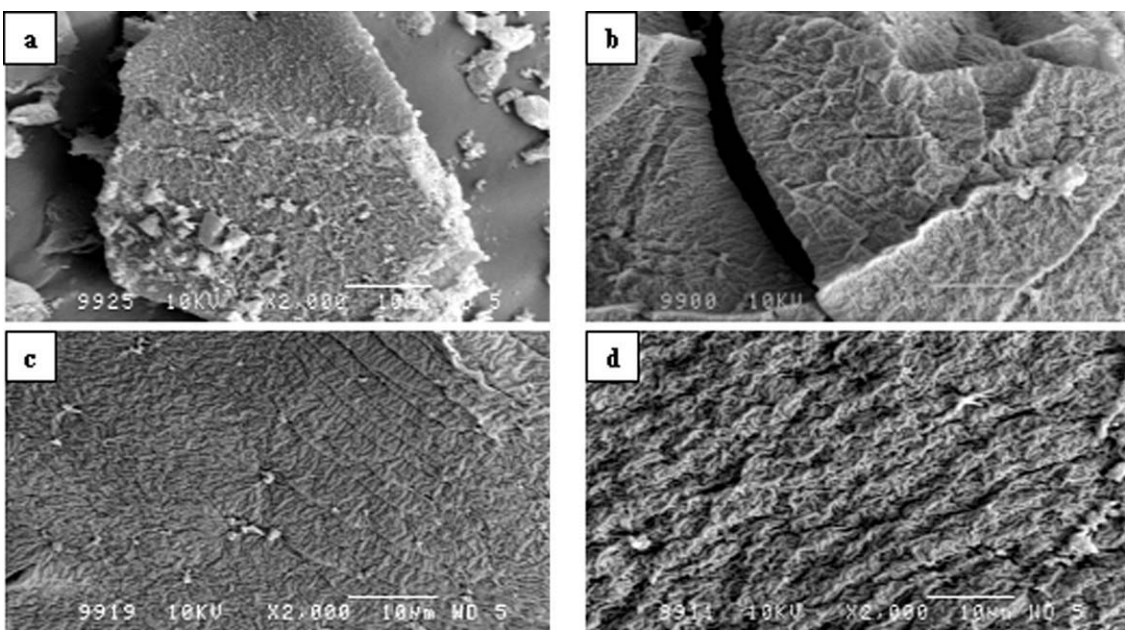


Photo 1 SEM of MMA-*co*-GMA and MMA-*co*-HEMA before and after cellulase immobilization. (a) MMA-*co*-GMA, (b) (MMA-*co*-GMA)-CMCT-cellulase, (c) MMA-*co*-HEMA, (d) (MMA-*co*-HEMA)-CMCT-cellulase.

TABLE I
Effect of Cellulase Concentration on Its Immobilization on MMA-*co*-GMA Via Covalent Attachment Method

Enzyme added (mg)	Enzyme added (U)	Enzyme immobilized (mg/ 0.1 g support)			Activity immobilized (U/ 0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.72	0.60	0.63	77.69	64.89	68.26	71.94	60.08	63.20
2	216	1.52	1.37	1.41	164.38	147.98	152.78	76.10	68.51	70.73
3	324	2.40	2.19	2.31	259.62	236.52	249.25	80.13	73.00	76.93
4	432	3.08	2.78	2.86	332.64	300.19	309.40	77.00	69.49	71.62
5	540	3.52	3.09	3.20	380.16	334.74	345.60	70.40	61.99	64.00

A = MMA-*co*-GMA, B = MMA-*co*-GMA with EGDMA (0.1%), and C = MMA-*co*-GMA with EGDMA (0.5%).

selection of support materials and the method of immobilization are very important for carrying out the desired enzymatic reaction. In the present study, a large variety of natural supports have been used as a carries for cellulase immobilization through covalent attachment and crosslinking methods as a two different techniques for the immobilization process.

Covalent attachment method

0.1 g of MMA-*co*-GMA and MMA-*co*-HEMA were used for immobilization of cellulase [from 1 to 5 mg (108–540 U)] in phosphate buffer pH 7 at 25°C for an incubation time 24 h. The maximum amount of cellulase activity was reached at concentration of 3 mg (324 U). The effect of cellulase concentration on the immobilization using the previously mentioned supports is shown in Table I and II. The retained activities were as follows: 80.13% and 67.91% for MMA-*co*-GMA and MMA-*co*-HEMA, respectively.

Crosslinking method

0.1 g of (MMA-*co*-GMA)-CMCT (0.1%), (MMA-*co*-GMA) with EGDMA (0.1% and 0.5%)-CMCT (0.1%), (MMA-*co*-HEMA)-CMCT (0.1%) and (MMA-*co*-HEMA) with EGDMA (0.1% and 0.5%)-CMCT (0.1%) were used for immobilization of cellulase [from 1 to 5 mg (108–540 U)] in phosphate buffer pH 7 at room temperature for an incubation time 4 h. The maximum amount of cellulase activity was reached at concentration of 3 mg (324 U). The effect of cellulase concentration on enzyme immobilized on the previously mentioned supports is shown in Tables III and IV. The retained activities were as follows: 91.92% and 74.63% for (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%), respectively. It can be observed from the earlier results that the highest values of the retained activities of the copolymers retention activities were in the absence of EGDMA.

Enzyme loading

From the earlier results, it was found that the amounts of bonded cellulose in case of covalent attachment method were 24.00 mg and 20.40 mg/g of MMA-*co*-GMA and MMA-*co*-HEMA, respectively. The preserved activities were found as 80.13% and 67.91% for the previous mentioned supports, respectively. On the other hand, the amounts of bonded cellulase in case of crosslinking method were 27.60 mg and 22.40 mg/g of (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%), respectively. The preserved activities were found to be 91.92% and 74.63% for the previously mentioned supports, respectively.

It was observed from the previous results that the activities of the immobilized cellulase on MMA-*co*-GMA and MMA-co-HEMA, crosslinked with CMCT (0.1%), are higher than those via covalent attachment method. This result may be due to that the crosslinker CMCT acts as spacer arms between the

TABLE II
Effect of Cellulase Concentration on Its Immobilization on MMA-*co*-HEMA Via Covalent Attachment Method

Enzyme added (mg)	Enzyme added (U)	Enzyme immobilized (mg/ 0.1 g support)			Activity immobilized (U/ 0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.58	0.48	0.52	62.43	52.19	56.27	57.81	48.33	52.10
2	216	1.25	1.09	1.14	135.00	118.37	123.12	62.50	54.80	57.00
3	324	2.04	1.80	1.93	220.03	194.69	208.07	67.91	60.09	64.22
4	432	2.42	2.10	2.21	261.14	227.02	238.98	60.45	52.55	55.32
5	540	2.82	2.36	2.53	304.24	254.72	273.46	56.34	47.17	50.64

A = MMA-*co*-HEMA, B = MMA-*co*-HEMA with EGDMA (0.1%), and C = MMA-*co*-HEMA with EGDMA (0.5%).

TABLE III
Effect of Cellulase Concentration on Its Immobilization on (MMA-*co*-GMA)-CMCT Via Crosslinking Method

Enzyme added (mg)	Enzyme added (U)	Enzyme immobilized (mg/ 0.1 g support)			Activity immobilized (U/ 0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.77	0.62	0.61	83.42	66.66	65.69	77.24	61.72	60.83
2	216	1.69	1.40	1.38	183.12	151.22	149.04	84.78	70.01	69.00
3	324	2.76	2.38	2.37	297.82	257.45	255.54	91.92	79.46	78.87
4	432	3.29	2.84	2.81	355.75	306.81	303.65	82.35	71.02	70.29
5	540	3.51	2.96	3.17	378.76	319.41	342.47	70.14	59.15	63.42

A = (MMA-*co*-GMA)-CMCT (0.1%), B = MMA-*co*-GMA with EGDMA (0.1%)-CMCT (0.1%) and C = MMA-*co*-GMA with EGDMA (0.5%)-CMCT (0.1%).

enzyme and the polymers, which decreases the steric hindrance. Accordingly the following investigations concerned with immobilized cellulase through crosslinking method.

Parameters affecting cellulase activity

The activities of free and immobilized cellulase were calculated by measuring the librated glucose at 540 nm, taking into account that the retention activities of the bounded cellulase on the different polymeric materials after immobilization process would be the initial activities (relative activity 100%) of them in the following parameters studied. The studying reactions were carried out at various pH values and temperatures, the effects of these parameters as well as storage stabilities and repeated use capabilities were examined.

Effect of pH. The pH dependence of the immobilized cellulase activity was compared with that of the free one in the pH range 3–10 at 25°C. Figure 5 shows the effect of pH on relative activity of cellulase in free and immobilized forms. It was found that the maximum activity was at pH 6 for immobilized cellulase on and (MMA-*co*-HEMA)-CMCT. Immobilized cellulase on chitosan-L-glutamic acid-GDA (1%) and (MMA-*co*-GMA)-CMCT have almost no activity variation (relative activity ~97% and 93%, respectively) under the pH range studied. The change in pH behavior after immobilization on carriers can be related to the free functional groups present in the reaction medium of the different polymers and enzyme after their reaction.[24]

It was observed that, at pH 10, the retained activities of the free and immobilized cellulase on (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%) were 93.14% and 74.90%, respectively. While at pH 3, the retained activities of the free and immobilized cellulase on the previously mentioned polymers were 93.25% and 84.61%, respectively.

Effect of temperature.. Figure 6 shows the effect of temperature on the activity of free and immobilized cellulase in the range 30–100°C at pH 7. The free cellulase exhibited a temperature optimum of 50°C, but it shifted to 70°C for immobilized on (MMA-*co*-GMA)-CMCT and 55°C for immobilized on (MMA-*co*-HEMA)-CMCT. Also it was observed that the temperature profile of the immobilized enzyme was slightly broader than that of the free one. The increase in optimum temperature was caused by changing the physical and chemical properties of the enzyme. These results demonstrate the effectiveness of the carriers protecting the enzyme activity under high temperature conditions.

It was observed that the free cellulase lost its all activity at 80°C, whereas at 100°C, the retained activities of the immobilized cellulase on (MMA-*co*-

TABLE IV
Effect of Cellulase Concentration on Its Immobilization on (MMA-*co*-HEMA)-CMCT Via Crosslinking Method

Enzyme added (mg)	Enzyme added (U)	Enzyme immobilized (mg/ 0.1 g support)			Activity immobilized (U/ 0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.61	0.57	0.52	66.04	61.57	56.08	61.15	57.01	51.93
2	216	1.38	1.28	1.19	148.80	138.84	129.21	68.89	64.28	59.82
3	324	2.24	2.11	1.97	241.80	227.09	212.61	74.63	70.09	65.62
4	432	2.69	2.69	2.33	290.17	280.80	251.19	67.17	65.00	58.32
5	540	2.96	2.93	2.50	320.22	316.22	270.22	59.30	58.56	50.04

A = (MMA-*co*-HEMA)-CMCT (0.1%), B = MMA-*co*-HEMA with EGDMA (0.1%)-CMCT (0.1%) and C = MMA-*co*-HEMA with EGDMA (0.5%)-CMCT (0.1%).

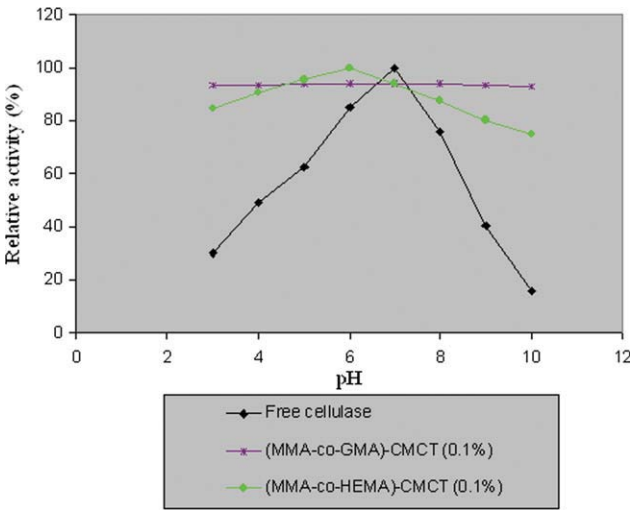


Figure 5 Effect of pH on the relative activities of free and immobilized α-amylase on (MMA-*co*-HEMA)-CMCT (0.1%) through crosslinking method. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

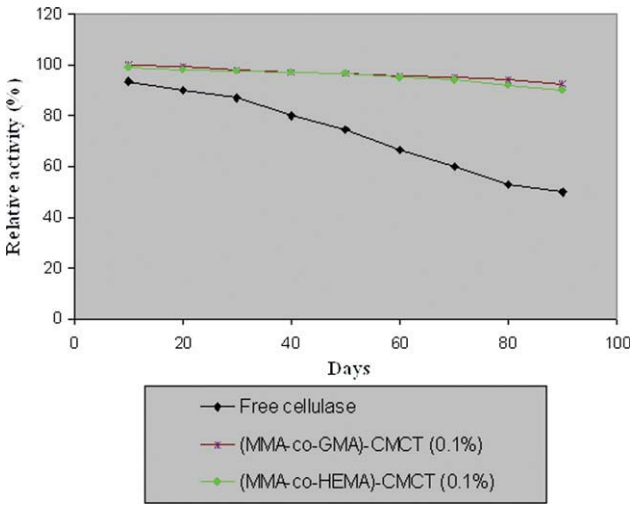


Figure 7 Storage stability of the free and immobilized α-amylase on MMA-*co*-HEMA)-CMCT (0.1%) through crosslinking method. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%) were 72.0% and 59.81% from their original activity, respectively.

Storage stability. Storage stability of immobilized enzymes is important for their practical application. The storage stabilities of free and immobilized cellulase in the dry state at 4°C for 90 days were investigated by measuring the cellulase activity at certain time intervals, and the results are given in Figure 7. The activity was determined at 25°C in phosphate buffer pH 7. It was found that the free cellulase maintained 50% from its original activity and the other retained activities for immobilized cellulase on (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%) were 92.61% and 90% from their initial activities, respectively, at the end of this period.

Repeated use capability. Immobilized cellulase was used repeatedly to hydrolyse CMC, and the reusability examined because of its importance for repeated industrial applications in a batch or a continuous operations. In this study, the activity of the immobilized cellulase was detected initially, and then it was washed thoroughly with phosphate buffer solution, and then used for the next activity measurement.

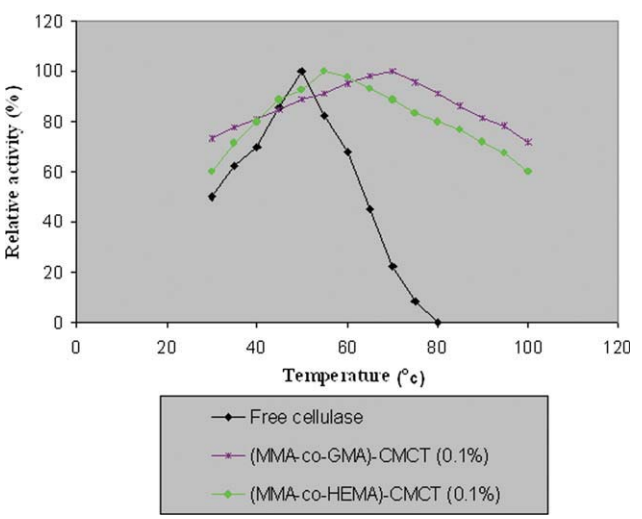


Figure 6 Effect of temperature on the relative activities of free and immobilized α-amylase on (MMA-*co*-HEMA)-CMCT (0.1%) through crosslinking method. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

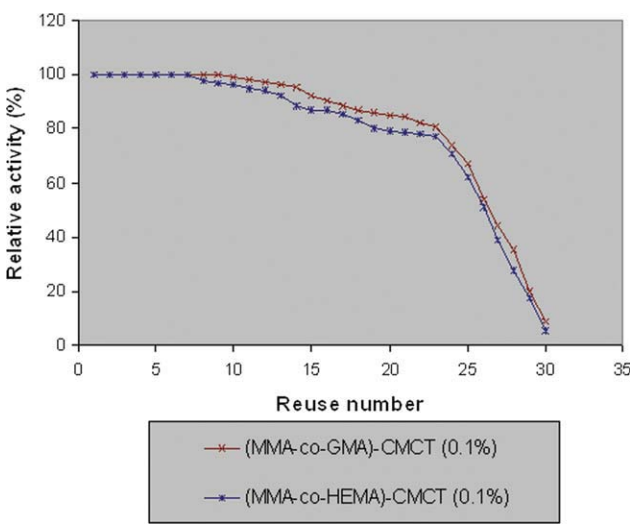


Figure 8 Reuse stability of the immobilized α-amylase on (MMA-*co*-HEMA)-CMCT (0.1%) through crosslinking method. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 8 illustrates the effect of repeated use of the activity of cellulase. It can be observed that the activities of the immobilized cellulase on (MMA-*co*-GMA)-CMCT (0.1%) and (MMA-*co*-HEMA)-CMCT (0.1%) did not change after 10 and eight times of repeated use and maintained 67% and 62% from their original activities after 25 times, respectively. In comparison, the activity retained by the immobilized cellulase using the previous mentioned polymers, in the present study, is higher than the previous studies.[25,26] These results indicate that the immobilized cellulase is relatively stable without severe loss of activity after several repeated uses.

CONCLUSIONS

The present study demonstrates that chitosan and its amino acids condensation adducts are promising carriers for the cellulase enzyme. The immobilization of cellulase on MMA-*co*-GMA and MMA-*co*-HEMA as a new matrix for cellulase immobilization by using covalent and crosslinking methods promoted cellulase stability, and as a result, the cellulase become more stable to pH, temperature, storage, and reuse compared with the free one.

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