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Preparation and Properties of Various Enzymes covalently immobilized on Polymethylglutamate

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Polymethylglutamate (PMG), a synthetic polypeptide, was used as a carrier to immobilize glucose oxidase (EC 1.1.3.4), uricase (EC 1.7.3.3), peroxidase (EC 1.11.1.7), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), urease (EC 3.5.1.5), and aminoacylase (EC 3.5.1.14) by the azide method. The enzymes could be immobilized covalently on PMG coated on glass beads. The retained activities of all the immobilized enzymes were excellent (more than 90%). The amount of enzyme immobilized on the polymer varied markedly depending on the kind of enzyme (trypsin 30 mg, chymotrypsin 27 mg, urease 5.8 mg, uricase 5.6 mg, aminoacylase 2.3 mg, glucose oxidase 1.8 mg and peroxidase 2.3 mg/100 mg PMG). It was found that the amount of bound enzyme was determined primarily by the molecular weight and secondarily by the content of lysine residues in the enzyme. The heat stabilities of the resulting immobilized enzymes were markedly improved, while the optimal pH and K_m values were almost unchanged. The enzymes immobilized on PMG showed improved stability because of both the increased hydrophilicity of the polymer and the multipoint binding mode, including covalent and ionic bonding.

Keywords——immobilized enzyme; polypeptide; acyl azide; glucose oxidase; uricase; peroxidase; trypsin; chymotrypsin; urease; aminoacylase

Introduction

Many methods have been applied for enzyme immobilization on many different polymers.[2] Hydrophilic and protein-like polymers are thought to be preferable as carriers for enzymes. Collagens,[3] copolymers of amino acids,[4] albumin modified with glutaldehyde[5] and elastin[6] have so far been used on this basis. Although these hydrophilic polymers overcome to some extent the drawbacks in enzyme immobilization, enzymes bound to the polymers lost significant amounts of activity, and the carriers were usually not in appropriate forms for practical use. We have therefore investigated the use of poly-γ-methyl-D-glutamate (PMG), a synthetic polypeptide, as an alternative carrier. Recently we reported that urease could be immobilized by the azide method on PMG in various forms, such as film, fiber and coating on beads or tubes, without significant loss of the activity, and the immobilized urease was found to retain more than 80% of its initial activity even after discontinuous use for a year.[7]

In this paper we describe studies on the use of PMG to immobilize various enzymes (urease, aminoacylase, trypsin, chymotrypsin, uricase, glucose oxidase, and peroxidase) in order to

1) Location: *1-1, Suzuki-cho, Kawasaki-ku, Kawasaki, 210, Japan.*
2) I. Chibata, "Immobilized Enzymes, Research and Development," Kodansha Ltd., Tokyo, 1978, pp. 10—73.
3) *a*) I. Karube and S. Suzuki, *Biochem. Biophys. Res. Commun.*, **47**, 51 (1972); *b*) S.S. Wang and W.R. Vieth, *Biotechnol. Bioeng.*, **15**, 93 (1973); *c*) P.R. Coulet, J.H. Julliard, and D.C. Gautheron, *Biotechnol. Bioeng.*, **16**, 1055 (1974).
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assess its feasibility as an enzyme carrier in comparison with other carrier polymers. The factors that contribute to the stabilization of the enzymes bound to PMG and the relationship between the amount of bound enzyme on PMG and the nature of the enzyme protein are discussed.

Materials and Methods

Materials

Poly-γ-methyl-D-glutamate (PMG), Ajicoat A-200, was supplied by Ajinomoto Co., Inc., Tokyo, Japan, as a 10% solution in ethylene dichloride. Silicon rubber adhesive, TSE-371, was purchased from Toshiba Silicon Co., Inc., Tokyo, Japan. Porous glass beads, CPG-10, were purchased from Electro-Nucleonics Inc., Fairfield, N.J., U.S.A. (150—180 μm in diameter). Trypsin (EC 3.4.21.4) (2×crystallized) and α-chymotrypsin (EC 3.4.21.1) (3×crystallized) were purchased from Worthington Biochemical Co., Freehold, N.J., U.S.A. Urease (EC 3.5.1.5) (115 U/mg), glucose oxidase (EC 1.1.3.4) (70, 210 U/mg) and peroxidase (EC 1.11.1.7) (115 U/mg) were purchased from Boehringer Mannheim-Yamanouchi K.K., Tokyo, Japan. Urease (1140 U/mg) was purchased from Sigma Chemical Co., St. Louis, U.S.A. Uricase (EC 1.7.3.3) (6.3 U/mg) was purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. Aminoacylase (EC 3.5.1.14) (0.6, 1.8 U/mg) was obtained from Amano Pharmaceutical Co., Nagoya, Japan.

Methods

Preparation of PMG Coated Glass Beads——First, 10 g of 10% PMG solution was mixed with 0.5 g of silicon rubber adhesive in 50 ml of chloroform. The mixed solution was then added to 10 g of porous glass beads. The solvent was removed in a rotary evaporator at 37° under reduced pressure. The glass beads with PMG were dried at 80—90° for 5 hr and were then allowed to stand for 3—5 days at room temperature.

Preparation of PMG Acyl Azides——PMG-coated glass beads were immersed in a mixture of 80% hydrazine hydrate and ethanol (1: 1) at 37° for 1 hr. The resulted hydrazides were filtered off and washed with methanol. The washed beads were then rinsed twice with 0.05 N hydrochloric acid, and with water until the pH of the washings became neutral. The content of hydrazide groups, which was determined by a method similar to that of Stolle,[8] was found to be 0.2 mg equivalent per 100 mg of PMG. Next, 100 mg of PMG containing hydrazide groups was immersed in 10 ml of ice-cold 0.1 N hydrochloric acid, then 2 ml of ice-cold 3% sodium nitrite was added dropwise. The mixture was stirred gently at 0° for 20 minutes. The beads were quickly separated by filtration and washed several times with ice-cold 50% ethanol, then rinsed once with the buffer solution used in the coupling reaction for each enzyme. The resulting PMG acyl azides were immediately used for the preparation of immobilized enzymes.

Coupling of Enzymes with PMG Acyl Azides——The PMG acyl azide beads were immersed in an ice-cold buffer solution containing 1—2 mg/ml of enzyme, and the mixtures were shaken gently at 0—5° for 1—2 days. Urease, aminoacylase, glucose oxidase and peroxidase were coupled in 0.1 M sodium phosphate and 2 mM EDTA (pH 8.7), and uricase was reacted in 0.1 M sodium borate (pH 8.9). These enzymes were dialyzed against the same buffer solution at 4° before the coupling reaction. In the cases of trypsin and chymotrypsin, the enzymes were added as crystalline powders to a suspension of the activated polymer in 0.1 M sodium phosphate (pH 8.7). The carrier-bound enzymes were separated by centrifugation and were washed several times with the appropriate buffer solution containing 1.0 M sodium chloride, until no enzyme activity could be detected in the washings. Urease, aminoacylase, glucose oxidase and peroxidase were washed with 0.1 M sodium phosphate and 2 mM EDTA (pH 7.0), and uricase was washed with 0.1 M sodium borate (pH 8.5). In the cases of trypsin and chymotrypsin, 0.002 N hydrochloric acid containing 1.0 M NaCl was used as a washing solution.

Assay of Enzyme Activity——Immobilized urease suspension (0.5 ml) was added to 5 ml of 0.25 M urea in 0.05 M sodium phosphate and 2 mM EDTA (pH 7.0). The mixture was allowed to react at 25° for 5.0 min with stirring. After the reaction period, 20 μl of the supernatant was taken up with a micro-pipet, and was mixed immediately with 0.5 ml of 0.05 N hydrochloric acid. The ammonium content in the solution was determined by the indophenol method.[9]

Immobilized trypsin suspension (0.2 ml) was mixed to 2 ml of 2 mM N-benzoyl-DL-arginine-*p*-nitroanilide in 0.1 M sodium phosphate (pH 8.0). The reaction was performed at 25° for 20 min with stirring. After adding 2 ml of 3% acetic acid, the *p*-nitroanilide concentration of the supernatant was measured at 410 nm. Immobilized chymotrypsin suspension (0.2 ml) was mixed with 2 ml of 0.25 mM N-benzoyl-L-tyrosine-*p*-nitroanilide in 0.1 M sodium phosphate (pH 8.0). The reaction was performed at 25° for 30 min with stirring. Nitroanilide in the supernatant was measured at 410 nm.

Immobilized aminoacylase suspension (1.0 ml) was added to 10 ml of 22 mM N-acetyl-DL-phenylalanine and 0.1 mM cobalt chloride in 50 mM veronal buffer (pH 8.0). The mixture was stirred at 37° for 30 min and

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0.2 ml of the supernatant was then kept for 5 min at 95°. Phenylalanine was determined by the colorimetric method of Yamm and Cocking.[10]

Immobilized glucose oxidase suspension (0.5 ml) was added to 10 ml of 100 mM D-glucose, 0.2 mg/ml 4-aminoantipyrine, 0.5 mg/ml phenol, 0.05% Triton X-100 and 0.02 mg/ml peroxidase (Sigma Chemical Co., type II) in 0.05 M sodium phosphate (pH 6.5). The mixture was stirred at 25° for 5 min. The dye produced in the supernatant was determined spectrophotometrically at 505 nm.

Immobilized uricase suspension (0.5 ml) was added to 5 ml of 0.1 mM uric acid and 2 mM EDTA in 0.1 M sodium borate (pH 8.0). The enzyme reactions were performed at 25° for 5 minutes with stirring. After adding 0.2 ml of 20% trichloroacetic acid to the reaction mixture, uric acid in the supernatant was determined at 292 nm.

Immobilized peroxidase suspension (0.5 ml) was added to 10 ml of 0.4 mM hydrogen peroxide, 0.2 mg/ml 4-aminoantipyrine, 0.5 mg/ml phenol and 0.05% Triton X-100 in 0.05 M sodium phosphate (pH 7.0). The mixture was stirred at 25° for 5 min. The dye in the supernatant was determined at 505 nm.

Determination of the Amount of Protein bound to the Polymer——The bound protein was estimated from the difference between the amount of protein used in the coupling reaction and that found in the washings. Protein concentration was measured by the method of Lowry *et al.*[11]

Results and Discussion

Preparation of Immobilized Enzymes

The azide method has already been applied for enzyme immobilization.[3c,12] Use of PMG as a carrier for immobilized enzymes may be justified by its protein-like features and its many methylester groups. The average molecular weight of the polymer used was 1.1×10^5 daltons in solution. Once the polymer is coated and dried, it is denatured and becomes insoluble except in strong acid solution. The thickness of the PMG coating was 3—5 μm, as estimated by scanning electron microscopy. The methylester residues on the surface of the PMG coating were converted to the acyl azide employing activation conditions milder than those of Mitz *et al.*[12a] Urease, aminoacylase, trypsin, chymotrypsin, uricase, glucose oxidase and peroxidase were thus immobilized covalently on the activated PMG. The results are summarized in Table I. It can be seen that almost all the activities of the enzymes used for the coupling reactions were immobilized when the enzymes were used in slight excess with respect to the carrier. On the other hand, when the enzymes were used in large excess relative to PMG, all of the excess enzyme was recovered in the washings without any significant loss of activity. The total activity yields for enzyme immobilization were quantitative in both cases. All the immobilized enzymes were found to exhibit specific activities as high as more than 90% of those of the intact enzymes. Trypsin and chymotrypsin were immobilized on the polymer

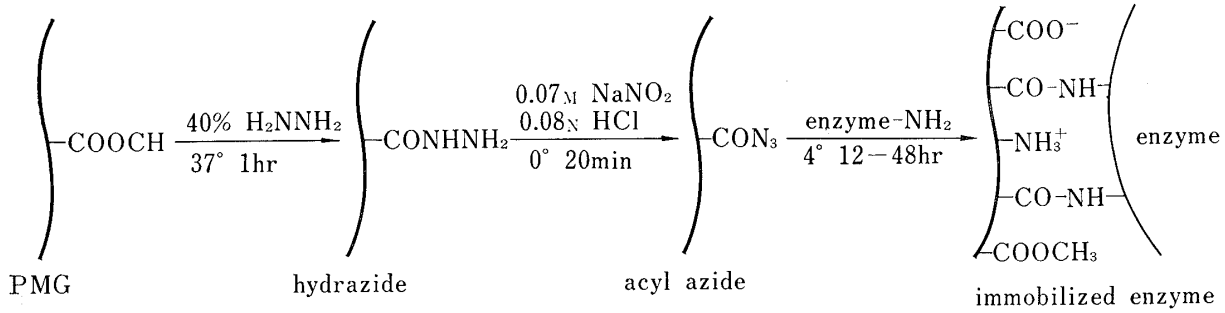

Fig. 1. Schematic Representation of the Procedure for Enzyme Coupling on Activated PMG, and of the Resulting Immobilized Enzyme

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Table I. Preparation of Various Enzymes Immobilized on PMG[a]

Enzyme	Enzyme activity U[b]; (%)			Specific activity bound/native enzyme (%)
	Added to coupling mixture	Bound on carrier	Recovered in washings	
Trypsin	25.5 (100)	25 (98)	0.2 (1)	98
Chymotrypsin	10.2 (100)	9.7 (95)	0.3 (3)	96
Urease	345 (100)	328 (95)	16 (5)	98
Amino acylase	4.3 (100)	1.7 (40)	2.5 (59)	92
Glucose oxidase	320 (100)	190 (60)	106 (33)	90
Uricase	18.6 (100)	17.3 (93)	0.9 (5)	95
Peroxidase	360 (100)	132 (37)	204 (57)	90

a) PMG (50 mg) coated on glass beads was used in each experiment.
b) The unit is the international unit.

with very high retained activities, although those proteases are known to be inactivated through autolysis.

Although they were covalently immobilized, the activities of all the enzymes used in this experiment were virtually completely retained. It is a well-known drawback of the covalent-binding method that enzymes are generally inactivated by immobilization on carriers. For example, the retained activities of urease[13] and uricase[5,6] immobilized covalently were not more than 80% and 50%, respectively. Aminoacylase could not be immobilized by the azide method on carboxymethylcellulose (CMC), and its activity was only 30—40% of that of the intact enzyme upon covalent binding on other carriers.[14] It was also reported that the retained activities of trypsin and chymotrypsin bound on CMC-azide,[15] a copolymer of ethylene and maleic acid-azide,[12d] and a copolymer of L-alanine and L-glutamic acid[16] were 90%, 40% and 43% of that of native trypsin and 18%, 13% and 38% of that of native chymotrypsin, respectively. Therefore, it is interesting that the specific activities of the covalently immobilized enzymes on PMG are nearly same as those of the native enzymes, not only in the cases of glucose oxidase and urease, which are relatively stable, but also in the cases of uricase and chymotrypsin, which are considerably labile.

These advantages of the present method seem to be due to the use of polypeptide as a carrier, and to the use of the mild azide method for coupling. Since the content of hydrazides on the polymer was 2—3 mg equivalent/g PMG, many acyl azide groups would be formed on the surface of the activated polymer. The excess acyl azide groups which could not couple to the enzymes would decompose into either carboxyl groups *via* hydrolysis or amino groups *via* Curtius rearrangement.[17] The infrared spectrum of PMG film after acyl azidation and drying for 30 minutes at 30° is shown in Fig. 2. In the spectrum, the strong and sharp peak at 2180 cm^{-1} and the other sharp peak at 2290 cm^{-1} were assigned to absorptions of the azide and isocyanate groups, respectively. The isocyanate peak increased in strength as the drying time increased. This observation indicates that Curtius rearrangement in fact occurred on the activated PMG. The surface of the resulting polymer may therefore increase in hydrophilicity and may also show the characteristics of amphoteric electrolytes. These carboxyl and amino groups formed on the polymer would further stabilize the bound enzymes by ionic

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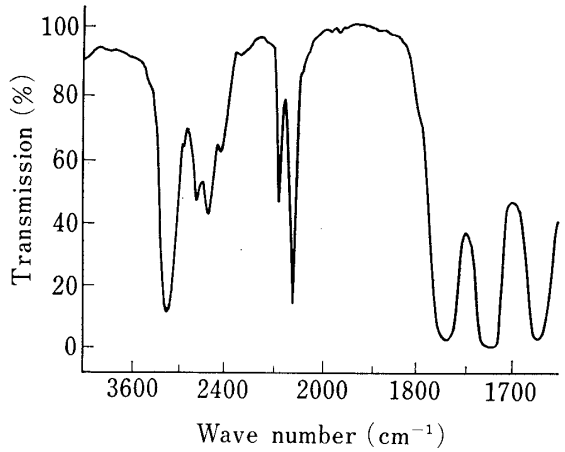

Fig. 2. Infrared Spectrum of PMG Acyl Azide Film

The infrared spectrum of PMG acyl azide film (2×3 cm, 30 μm thick) was taken directly with a JASCO IR-S recording spectrophotometer.

Table II. Preparation of Various Enzymes Immobilized on PMG: Correlation between the Amount of Bound Enzyme and the Molecular Weight of the Enzyme

Enzyme	M.W. $\times 10^3$	Amount of bound enzyme per 100 mg of PMG[b] (mg)
Trypsin	24	30
Chymotrypsin	25	27
Urease	480	5.8
Uricase	130[a]	5.6
Amino acylase	100[a]	2.3
Peroxidase	40[a]	2.3
Glucose oxidase	150	1.8

a) The molecular weight of the enzyme was estimated by gel filtration.
b) Mean value of three preparations.

interactions. Taking these considerations into account, the enzymes are thought to be bound to the polymer by multi-point binding (Fig. 1). In this respect, the present method can be considered to represent a marked improvement in the preparation of immobilized enzymes, especially unstable enzymes.

The amount of enzyme immobilized on the polymer varied markedly depending on the kind of enzyme. The relationships between the amount of the immobilized enzyme and the molecular weight, the specific activity and the amino acid composition of the enzyme were examined. As shown in Table II, enzymes with a smaller molecular weight seem to be much more effectively immobilized on the polymer. On the other hand, the specific activity of the immobilized enzyme was almost the same as that of the original enzyme, regardless of its purity (Table III). Therefore, the enzyme and contaminating proteins of similar molecular

Table III. Preparation of Various Enzymes Immobilized on PMG: Correlation between the Amount of Bound Enzyme and the Specific Activity of the Native Enzyme

Enzyme	Specific activity native enzyme (U/mg)	Amount of bound enzyme/ 100 mg PMG (mg)	Specific activity Bound/ native enzyme (%)
Urease	115	5.8	98
	1140	5.6	97
Aminoacylase	0.6	2.1	92
	1.8	2.3	95
Glucose oxidase	70	1.5	91
	210	1.8	90

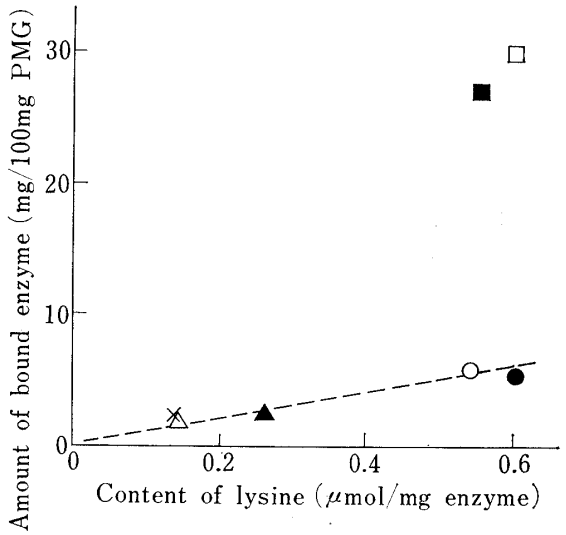

Fig. 3. Correlation between the Amount of Bound Enzyme on PMG and the Content of Lysine Residues in the Enzyme

□: trypsin, ■: chymotrypsin, ○: urease, ●: uricase, ▲: aminoacylase, ×: peroxidase, △: glucose oxidase.
Amino acid compositions of enzymes were determined with a Hitachi KL-5 amino acid analyzer after hydrolysis with 6 N hydrochloric acid at 110° for 24 hr.

weight were considered to be nonspecifically immobilized. With respect to the enzyme amino acid composition, it was observed that the amount of bound enzyme was correlated with the content of lysine residues in preparations with an enzyme molecular weight higher than 10^5 daltons (Fig. 3). Although acyl azides of CMC are known to react with lysine, cysteine, tyrosine, serine and threonine,[18] there was no correlation with the contents of other amino acid residues. These results suggest that the acyl azide groups on the polymer couple mainly with lysine residues. It can be considered that the amount of immobilized enzyme is determined primarily by the molecular weight of the enzyme and secondarily by the content of lysine residues in the enzyme preparation.

Only a few micrograms of enzymes have been immobilized per 25 mg of collagen in which the carboxyl groups were converted to acyl azide groups.[3c] Therefore PMG can immobilize far larger amounts of enzymes than collagen, even though both carriers are polypeptides and the same azide methods is employed.

Catalytic Properties of Enzymes Immobilized on PMG

The apparent optimal pH's and K_m values of the native and immobilized enzymes are summarized in Table IV. The apparent optimal pH's and K_m values of the immobilized enzymes on PMG were almost unchanged from those of the intact enzymes. There was apparently little effect of the immobilization on the catalytic properties of the enzymes. It has been reported that the optimal pH's of bound enzymes tend to change depending on the surface charge of the carrier,[19] and that the changes in apparent K_m values may by caused by interference in the diffusion of substrate or product inside the carriers.[20] The above results may therefore indicate that the enzymes are immobilized on the exterior surface of the PMG, and that the overall charge around the bound enzymes is neutral.

Table IV. Optimal pH and K_m Values of Native and PMG–Immobilized Enzymes

Enzyme	Substrate	Optimal pH		K_m (mM)	
		Native	Bound	Native	Bound
Trypsin	BApNA[a]	8.5	8.8	0.86	0.71
Chymotrypsin	BTpNA[b]	8.2	8.3	0.21	0.18
Urease	Urea	7.1	7.2	11	16
Amino acylase	Ac–DL-Phe[c]	7.2	7.1	2.3	2.1
Glucose oxidase	D-Glucose	5.3	5.5	9.0	12
Uricase	Uric acid	8.2	8.5	0.020	0.022
Peroxidase	H_2O_2	7.3	7.0	—	—

a) N-Benzoyl-DL-arginine-*p*-nitroanilide.
b) N-Benzoyl-L-tyrosine-*p*-nitroanilide.
c) N-Acetyl-DL-phenylalanine.

Although trypsin and chymotrypsin immobilized on PMG retained very high activities towards low-molecular substrates, they exhibited activities for casein as low as 7% of that of intact trypsin and 5% of that of intact chymotrypsin, respectively. This supports the view that trypsin and chymotrypsin bound on the polymer were not denatured, but were not accessible to high molecular substrates, because of the steric hindrance around the enzyme molecules.

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20) R. Axén, P.A. Myrin, and J.C. Janson, *Biopolymers*, **9**, 401 (1970); T. Mori, T. Tosa, and I. Chibata *Biochim. Biophys. Acta*, **321**, 653 (1973).

Stability of Immobilized Enzymes on PMG

Heat stabilities of the native and immobilized enzymes are summarized in Table V. As is evident from Table V, the heat stabilities of all the enzymes examined were markedly improved by immobilization. The enhancement of heat stability was high compared to those of immobilized enzymes previously reported. It is interesting that the heat stability of all of the enzymes could be enhanced by immobilization on PMG.

Table V. Heat Stability of Native and PMG–Immobilized Enzymes

Enzyme	Treatment			Remaining activity[a] (%)	
	Temp. (°C)	Time (min)	pH	Native enzyme	Immobilized enzyme
Trypsin	55	30	8.0	6	82
Chymotrypsin	55	30	8.0	9	76
Urease	75	30	7.0	12	67
Amino acylase	70	30	7.2	11	73
Glucose oxidase	65	30	6.0	10	65
Uricase	55	30	8.5	5	75
Peroxidase	60	30	6.5	12	68

a) Determined under standard conditions after the heat-treated enzyme had been rapidly cooled.

As regards the stability of bound enzymes, it is likely that the enzymes are stabilized by immobilization on the carrier in a multi-point binding mode.[21] As the enzymes are bound on the surface of PMG, which contains acyl azide, carboxyl and amino groups, it can be considered that the bound enzyme molecules would further react with these functional groups. These secondary interactions, which include covalent and ionic bonding, would lead to a multipoint binding mode (Fig. 1). This would tend to give enzymes immobilized by this method improved stability.

In view of the improved retained activities and stabilities, PMG appears to be a versatile carrier for various enzymes.

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