

Studies on the Preparation of Cross-Linked Polyvinylpyrrolidone-g-Poly (Maleic Anhydride-co-Styrene) and the Immobilization of Ovalbumin on the Graft Copolymer

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Abstract. Cross-linked polyvinylpyrrolidone (PVPy), irradiated in a γ -chamber was grafted with poly (maleic anhydride-co-styrene). Percentage of grafting was studied as a function of various reaction parameters. The maleic anhydride residues of the graft copolymer offer reactive sites for designing polymeric reagents. In the present communication, we report the immobilization of ovalbumin on the graft copolymer.

1. Introduction

Cross-linked poly (N-vinylpyrrolidone), PVPy in beaded form is prepared by suspension polymerization of N- vinylpyrrolidone along with a cross-linking agent¹⁻⁵. PVPy is one of the most non-toxic polymers known. PVPy swells both in aqueous and polar organic media, a property that makes it an excellent support for developing into a reagent for organic synthesis.

PVPy irradiated in air from a γ - source when heated with a mixture of maleic anhydride and styrene is grafted with an alternating copolymer of maleic anhydride and styrene. A detailed study has been made of grafting under a variety of reaction parameters. The residues of maleic anhydride offer reactive sites for further chemical transformation and designing polymeric reagents. The present communication reports the preliminary studies on the use of the graft copolymer for the immobilization of a protein tagged to the insoluble matrix by the amino group (α or ϵ -) of the protein. Immobilized enzymes serve as polymer-supported catalysts. Immobilized protein is used in solid phase sequencing. Immobilization finds use in affinity chromatography.

2. Experimental

General procedure of graft copolymerization : Samples of PVPy irradiated in air in a γ -chamber were suspended in dioxane containing a known composition of a mixture of maleic anhydride and styrene. The reaction mixture was refluxed in an oil

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bath. Similar experiments were run in benzene or toluene or distilled water or dioxane water (1:1). The graft copolymer was freed from the soluble copolymer by washing. The percentage of grafting was calculated from the increase in initial weight of PVPy.

Percentage of grafting was studied as a function of total dose (Fig. 1), reaction time (Fig. 2) and monomer concentration (Fig. 3). With respect to reaction media it was found that there was no grafting in benzene, toluene and water. However, Table I presents the results of grafting in dioxane and aqueous dioxane.

Table I.

Total dose (MR)	0.48	1.44	1.92	2.4	3.36	3.84	4.32	208
% Grafting *	152	188	240	278	340	302	259	208
% Grafting **	494	578	665	726	800	744	679	510

* Medium : Dioxane(3ml) Temperature : 1350 C % Grafting; **Medium Dioxane:Water (1.5ml:1.ml)
Temperature : 1300C

To study the percentage of grafting of maleic anhydride (in water) at 120⁰ and of styrene (in dioxane) at 135⁰ on PVPy, experiments were run with increasing preirradiation doses (1.44 MR to 3.84 MR). There was no grafting.

Percentage of grafting was also studied by varying the composition of the mixtures of maleic anhydride and styrene taken as monomers. The total amount of monomers was kept constant at .020 mole for each reaction. Table II presents the results.

Table II. Graftin with variation in composition of mixture of maleic anhydride and styrene (PVPy, 100mg; total dose, 3.36 MR; dioxane, 3ml, temperature, 135⁰ C, reaction time, 3h)

Maleic anhydride (moles)	Styrene (moles)	% Grafting
0.0	0.020	0
0.003	0.017	125
0.005	0.015	211
0.007	0.013	298
0.01	0.01	340
0.013	0.007	291
0.015	0.005	205
0.017	0.003	114
0.020	0.0	0

Evidence of Grafting : IR spectrum (KBr pellet) of the grafted polymer showed peaks at 3050 cm⁻¹ for Ar-H stretching, at 1600 cm⁻¹ for C=C stretching and at 710 cm⁻¹ for C-H deformation vibrations, indicating that styrene has been grafted onto PVPy beads. Peaks also appeared at 1850 cm⁻¹ due to C = C stretching and the doublet due to the coupled vibrations of two C=O groups confirming the grafting of maleic anhydride onto PVPy.

Study of Swelling of PVPy in Different Media : PVPy beads (100mg) were left to swell for 6h in water, in dioxane and in dioxane-water (1:1) and the bed volumes were noted. Bead volumes of dry PVPy beads (100mg; 0.21ml) on swelling were found to be 0.58ml, 0.42 ml and 0.50ml in the above media respectively.

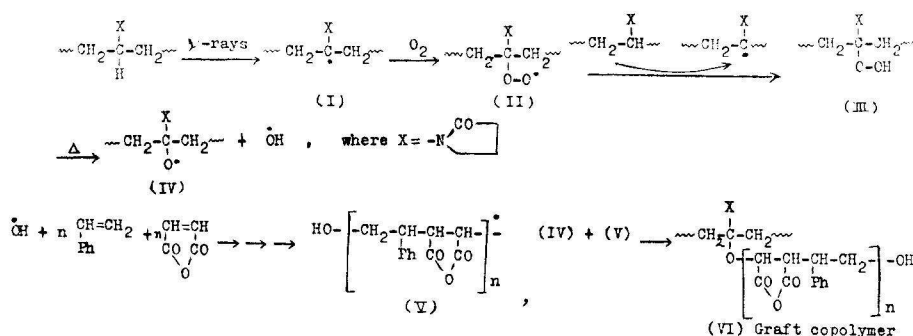
Immobilization of Ovalbumin on PVPy-g-poly (maleic anhydride-co- styrene) : Ovalbumin (200 mg) was dissolved in bicarbonate buffer (0.25M, 20ml) pH=10.01. PVPy-g-poly (maleic anhydride-co- styrene) with 100% grafting (100mg) was added to the protein solution. Reaction mixture was gently stirred at r.t. for 3h and stored overnight. The polymer was filtered and washed with the buffer (3X), water (3X), alcohol and finally with acetone and dried at 50°C (140 mg).

To 20mg of graft copolymer-bound protein taken in a test tube, two drops each of 'Kaiser reagent' A, B and C were added. The tube was heated for 5 min. In a boiling water bath. Isopropanol (3ml) was added and the solution was filtered. Ovalbumin (20mg) and Resin (20mg) were similarly treated. At 570 nm on a spectrophotometer 'Spectronic-20' the absorbance recorded was 0.234, 0.82 and 0.01 for resin bound ovalbumin, ovalbumin +resin respectively.

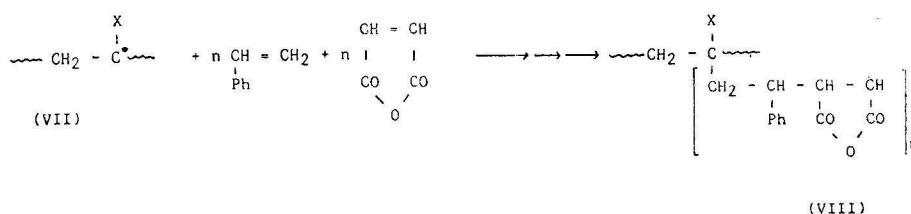
3. Discussion

Percentage of grafting increases linearly with an increase in the preirradiation dose, reaches a maximum at a total dose of 3.36 MR (both with 0.005 mole and 0.01 mole each of maleic anhydride and styrene) (Fig. 1). With the increase in total dose there is an enhancement in the formation of active sites thereby increasing the grafting. Beyond the optimum, irradiation causes excessive production of hydroxyl radicals and overcrowding of radical sites on the backbone polymer⁶. Under this condition the percentage of grafting is reduced.

General Mechanism of grafting

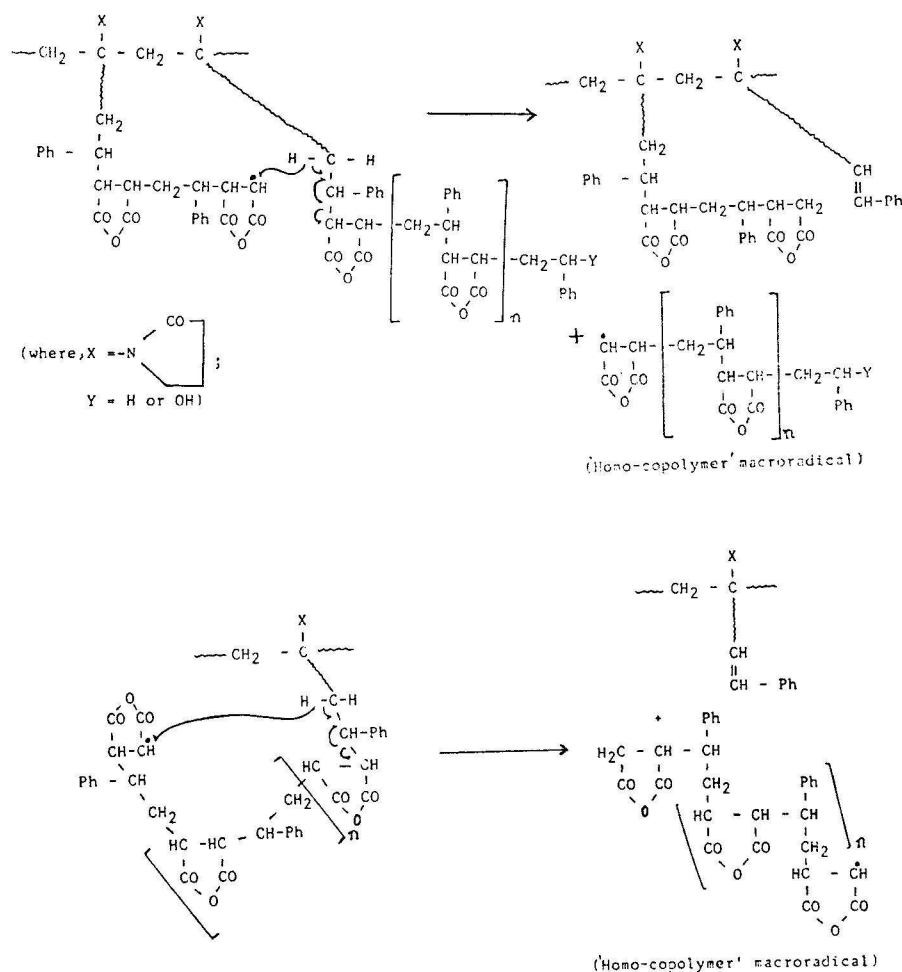


Grafting also occurs by initiation through radical sites on the polymeric backbone (Radical VII).



Percentage of grafting was studied as a function of total dose in benzene at 105°C, toluene at 140°C, distilled water at 120°C and in dioxane : water (1:1) at 130°C where the heterogenous mixture gently boiled to keep it stirred Table I presents the results. There was no grafting in water due to the fact that although the polymer PVPy swells very well in water, styrene is insoluble and maleic anhydride, although soluble, is incapable of grafting alone. There was no grafting in benzene or toluene which neither swell the PVPy beads nor dissolve maleic anhydride. Solution of styrene in benzene or to toluene cannot approach the radical sites on the polar beads. All these factors strongly negate grafting. There was considerable grafting in dioxane which increased more than double when dioxane : water (1:1) was chosen as the reaction medium. PVPy beads swell 2.8 times of its volume in water, 2.0 times in dioxane and 2.4 times in dioxane : water (1:1). A mixture of dioxane and water swells the beads more than dioxane and dissolves the monomers and poly (styrene-maleic anhydride) radicals, thus serving the requirements for a close contact between the reacting species reasonably well.

There was regular increase in the percentage of grafting as the time of reaction is increased from 60 min to 180 min beyond which it showed a decreasing trend (Fig. 2). A minimum was observed at 240 min. after which it continued to rise upto 300 min. of heating. The initial rise upto 180 min. appears to be a kinetic factor when grafting at the rate 15 mg/g PVPy /min. increases the weight of the graft copolymer linearly. When the chains grafted through the initiation of radical sites on PVPy are sufficiently long, they become flexible enough to attack themselves or neighbouring chains by abstracting a hydrogen atom, resulting in the cleavage of grafted chains and reduction in the percentage of grafting. This 'back-biting'⁷ shortens the length of the grafted chains and generates 'homo-copolymer' macroradicals and olefins (see Fig.2)



By this time it is expected that the monomer species are largely used up and the viscosity of the medium has increased considerably. Any grafting that now occurs proceeds through chain transfer and occurs at the outer surface of the PVPy beads on any residual radical sites. The mobility of 'homo-copolymer' macroradicals in the viscous medium is low and their diffusion into the interior of the polymer beads is not possible. All these factors are responsible for a slow rate of grafting (5.8 mg/g PVPy/min.) after 240 min.

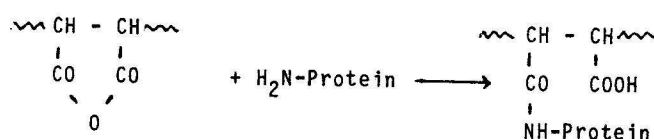
Keeping total dose fixed at 3.34 MR, percentage of grafting increased regularly with increasing monomer concentration from 0.003 mole to 0.020 mole each of maleic

anhydride and styrene (Fig.3.) Beyond this concentration excessive homopolymerization makes the work-up very difficult. Higher concentration of monomers is obviously a favourable condition for increased grafting because it should promote the chances of interaction between the reactants (radical sites and monomer or 'homo-polymer' radicals). As the concentration of the monomers is increased, more of the 'homo-copolymer' species are formed increasing the viscosity of the medium and restricting the movement of the monomer molecules and 'homo-copolymer' radical species. This results in their reduced availability at the radical sites of PVPy and causes a lower grafting. Fig. 3 illustrates the same trend.

Five experiments run with increasing dose of radiation with maleic anhydride alone as monomer in water at 120°C and with styrene alone as the monomer in dioxane at 135° C did not give any grafting. Maleic anhydride is known not to polymerize alone by free radical initiation and this explains the failure in grafting. We, however, have no explanation for the failure in grafting with styrene on PVPy.

Percentage of grafting was also studied with variations in the composition of mixtures of maleic anhydride and styrene on PVPy irradiated with a fixed total dose of 3.34 MR and the total of the monomers as 0.02 mole. Table II shows that grafting was maximum when an equimolar amount of each was used in agreement with the known formation of alternating polymer between maleic anhydride and styrene which utilize equimolar amounts of the two monomers.

PVPy-g-poly (maleic anhydride-co-styrene) with 100% grafting possesses succinic anhydride residues which react with amino groups of a protein (α - or ϵ -) at pH 10.01, by succinylation. The protein is thus immobilized on the resin.



We have immobilized ovalbumin on the graft copolymer. Ninhydrin colour reaction with Kaiser reagent proves the existence of protein on the conjugate. On estimation of absorbance at 570 nm, it is concluded that most of the amino groups of protein are still free showing thereby that multiple contact between the succinic anhydride residues and amino groups does not take place and the polymeric reagent indeed acts to isolate the reaction sites.

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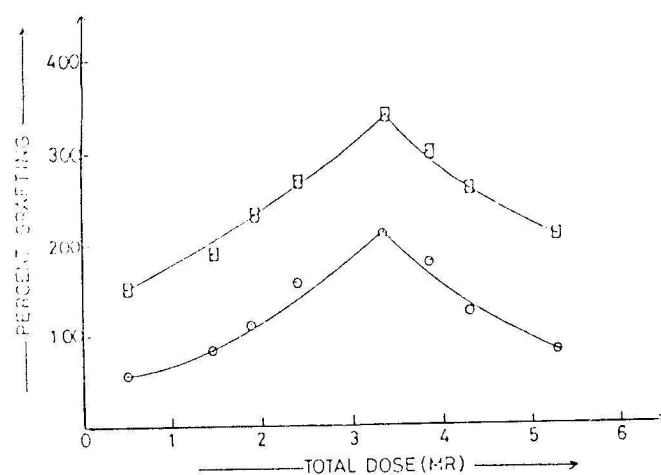


Fig. 1. EFFECT OF TOTAL DOSE ON THE PERCENTAGE OF GRAFTING OF MALEIC ANHYDRIDE AND STYRENE ON PVPy (PVPy, 100 mg, DIOXANE, 3 ml, REACTION TIME, 3h, TEMP, 135°C)

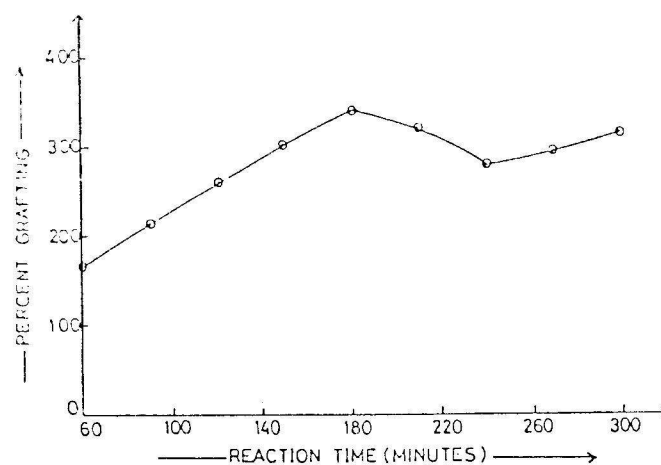


Fig. 2. EFFECT OF REACTION ON THE PERCENTAGE OF GRAFTING OF MALEIC ANHYDRIDE AND STYRENE ON PVPy (PVPy, 100 mg, MALEIC ANHYDRIDE AND STYRENE, 0.01 mole; DIOXANE, 3 ml; TEMP., 135°C; TOTAL DOSE, 3.36 MR).

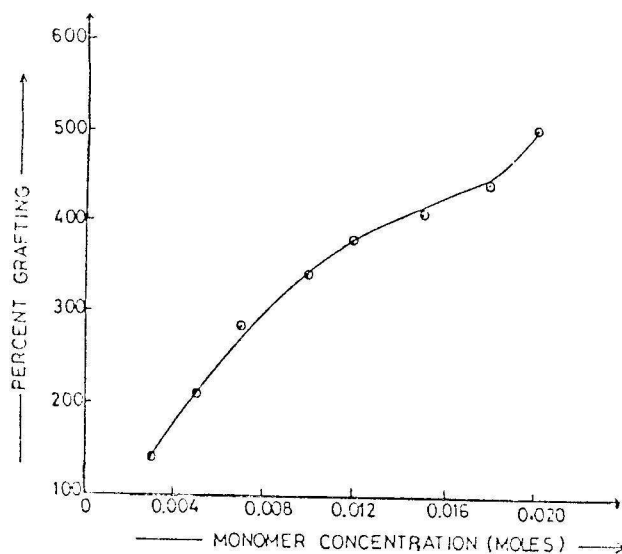


Fig. 3. EFFECT OF CONCENTRATION OF AMLEIC ANHYDRIDE AND STYRENE ON GRAFTING ONTO PVPy. (PVPy, 100 mg; DIOXANE, 3 ml; TEMP., 135°C; TOTAL DOSE, 3.36 MR; REACTION TIME, 3h).

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