MANGANESE INITIATED GRAFT COPOLYMERISATION OF VINYL MONOMERS ONTO COLLAGEN

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Graft copolymerisation of vinyl monomers such as methyl methacrylate (MMA), methyl acrylate (MA) and acrylonitrile onto collagen has been carried out using manganic ion as initiator. Collagen vinyl graft coplymers were characterised after acid hydrolysis of the collagen backbone with 6N HCl. The molecular weights of the grafted vinyl polymeric chains were in the range of 2.89 to 18.32×10^5. These values are in agreement with those obtained using Ce^{4+} initiation technique. The rate of grafting was in the order MMA>MA>AN. The number of grafting sites obtained from the molecular weight data indicated that the molecular weights of the isolated grafts are many times larger than the molecular weights of the tropocollagen molecules and the grafting sites are very few. These results are in agreement with those obtained by Ce^{4+} system. Proof of grafting has been provided by the I.R. spectra of the isolated grafted chains which showed characteristic amide absorption bands of the amino acids. Electron microscopy of the graft copolymers indicated the absence of cross-striations. A mechanism for the initiation copolymerisation using Mn^{3+} ion has been suggested.

In recent years, the formation of graft copolymers of collagen has received considerable attention. In previous papers,[1-9] we reported from this laboratory the ceric ion initiated graft copolymerisation of vinyl monomers onto collagen. The vinyl type of polymerisation is usually initiated by a free radical. Most graft polymers of collagen have been prepared therefore by creating free radical centers on the collagen backbone. This may be done by the application of high energy such as γ-rays or low energy such as ultraviolet light and visible light in the presence of certain sensitizers.[10-13] Many other activating agents have also been used successfully, e.g. Fentons' reagent,[14,15] vanadium[16] and potassium persulphate.[17-18] In the presence of a reactive backbone (double bonds or halogens) as in PVC or chlorinated rubber, even a catalyst like benzoyl peroxide may be used for grafting.[20,21] In this paper, we report the suitability of manganese ions to initiate graft copolymerisation of vinyl monomers onto collagen. Our reason for carrying out these studies was aimed at producing artificial reinforcement of collagen in hides and skins which could lead to the production of functionally modified leathers.

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Sing *et al*[22] and Namasivayam *et al*[23] have recently reported that manganic sulphate in excess sulfuric acid may form an effective redox system for grafting of polymethyl methacrylate on cellulose and polyvinyl alcohol. Since collagen is known to contain various functional groups such as hydroxyl, amino and imino which may be capable of forming a redox system with manganic ions, it was of interest to study the grafting of vinyl monomers on collagen using this system and to compare the sample with the previously studied ceric ion system.

Experimental

Materials

Collagen

Collagen prepared from the middle corium of buffalo hide was used as the source of insoluble collagen.

Monomers

Methyl methacrylate (MMA) and methyl acrylate (MA) were obtained from Rohm & Haas, USA; acrylonitrile (AN) from BDH. All were purified by standard methods as described in previous papers.

Enzyme

Pronase, B grade (Cal-Biochem; USA) was used without further purification.

Manganic sulphate

Manganic sulphate was prepared by oxidising manganous sulphate in sulphuric acid with potassium permanganate using the method described by Ubbelohde.[24]

Graft copolymerization procedure

The graft copolymerisation reactions were carried out in a round-bottomed three-necked flask of one litre capacity fitted with a water-sealed glass stirrer, a gas outlet and a thermometer. All the grafting experiments were carried out using stirring speeds of 150-200 r. p. m. at room temperature. In a typical experiment, 10 g. hide powder were dispersed in 400 ml. of distilled water. After oxygen-free nitrogen gas was bubbled through the reaction mixture to expel the air inside the flask for 30 minutes, the requisite amount of monomer was added. The amount of manganic sulfate solution calculated to give the desired concentration was then added. The reaction was allowed to proceed for 3 hours after which the resulting products were separated by filtration, washed with distilled water and extracted with the appropriate solvents of the homopolymers to remove the occluded and loosely bound homopolymer. Acetone in the case of MMA and MA grafted collagen and N,N-dimethyl formamide in the case of AN grafted collagen were used for extracting the homopolymers.

Analysis of the grafted products

The extracted, dried product was then analysed for total nitrogen, arginine and hydroxyproline as reported earlier.[3,4] The collagen content of the graft copolymers was calculated from the content of hydroxyproline (by multiplying the hydroxyproline content by 7.4)[4] or from the content of total nitrogen (by multiplying the nitrogen content by 5.6)[4] or from the arginine value (by multiplying the arginine content by 11.6).[4]

Characterization

Isolation of grafted vinyl polymer side chains

The grafted vinyl polymer side chains were isolated from collagen backbone by hydrolysis with hydrochloric acid and also by digestion with pronase using the procedure given in our earlier papers.[1-3]

Molecular weight of grafted vinyl polymer side chains

The viscosity and molecular weights of grafted vinyl branches isolated by acid hydrolysis were determined using a PCL suspended Level Dilution Viscometer. The intrinsic viscosities $[\eta]$ were obtained by plotting $\eta sp/C$ against C and extrapolating the straight line to zero concentration. Relationships used for the evaluation of molecular weights(M) were those due to Fox *et al*[25] for poly (methyl methacrylate) at 30°C in benzene

$$[\eta] = 8.69 \times 10^{-3} M_n^{0.76}$$

for poly (methyl acrylate) at 35°C in benzene due to Sen *et al*[26]

$$[\eta] = 1.282 \times 10^{-4} M^{0.819}$$

and for poly-acrylonitrile

$$[\eta] = 2.43 \times 10^{-4} \bar{M}_v^{0.75}$$

due to Cleland *et al*[27] at 25°C in dimethyl formamide

Infra-red spectra

To provide proof of grafting the infra-red spectra of the grafts isolated by acid and enzymatic hydrolysis were measured with a Perkin-Elmer Model 337 grating infra-red spectrophotometer using potassium bromide (KBr) pellets (500 mg.) containing 2-6 mg. of powdered polymers.[4,28]

Electron microscopy

To gain knowledge of the location of the grafted polymer in the collagen fibrils, electron microscopic observation of the grafted collagen fibrils was undertaken. Samples of collagen powder grafted with poly (MMA) were used for this purpose using the same procedure as reported in earlier paper.[4]

Percent grafting and the number of grafting sites

The percent grafting and the number of grafting sites per mole of collagen were determined as reported in the previous papers.[4,28]

Total conversion, the efficiency of grafting and the rate of grafting were calculated as follows :

$$\text{Total conversion}(\%) = \frac{\text{Weights of polymer grafted and homopolymer}}{\text{Weight of monomer charged}} \times 100$$

$$\text{Efficiency of grafting}(\%) = \frac{\text{Weight of polymer grafted}}{\text{Weights of polymer grafted and homopolymer}} \times 100$$

The rate of grafting in %/hr. was converted to R_p by use of the equation

$$\text{Rate of grafting } (R_p) = \frac{\%/\text{hr.}}{100} \times a \times \text{mole weight of monomer} \times \frac{1}{3600} \text{ mole/l/sec.}$$

where 'a' is the number of grams of collagen per litre of monomer-water-collagen.

Results and discussion

Grafting with different monomers

The results obtained (Table I) show that monomers such as methyl methacrylate, acrylonitrile and methyl acrylate are polymerised readily onto collagen using the Mn^{3+} ion initiation technique. Even though grafting took place in all cases, the different vinyl monomers were grafted onto collagen with various degrees of success. The percent grafting was found to be higher in the case of MMA as compared to AN

TABLE I

COMPOSITION OF COLLAGEN VINYL GRAFT COPOLYMERS[a]

Graft copolymer	Collagen in the graft (%)				Synthetic polymer in the graft (%)				Grafting[b] (%)
	By total nitrogen	By weight difference	By Argi-nine	By Hydroxy-proline	By total nitrogen	By weight difference	By Argi-nine	By Hydroxy-proline	
Collagen-PMMA[c]	40.54	40.55	40.34	30.72	59.46	59.45	59.66	69.28	146.7
Collagen-PMMA	45.60	45.91	46.38	39.58	54.40	54.09	53.62	60.42	119.3
Collagen-PMA	65.20	65.46	65.83	48.88	34.80	34.54	34.17	51.12	53.38
Collagen-PAN	—	60.95	59.25	38.30	—	39.05	40.75	61.70	51.88

a. Initiator concentration = 3.12×10^{-3} mole/l; temp. = 30°C; monomer = 20g.; time = 3 hrs.

b. Calculated on the basis of nitrogen values

c. In the absence of Na_2SO_4

and MA. These results were similar to the results of Ce^{4+} initiated grafting.[5] The lower percent grafting in the case of AN may be attributed to the complexing of Mn^{3+} and Mn^{2+} with the nitrile groups which reduced the available Mn^{3+} ions for the grafting reaction; on the other hand, the lower percent grafting of MA may be due to its low reactivity. Factors such as permeability of collagen to monomers, interaction of monomers to collagen and initiator etc., also contribute to variations in the degree of grafting. However, for specific monomers certain initiators were more effective than others in promoting polymerisation and/or grafting. These results were in accordance with those obtained using ceric ion initiation.[5] Since the grafting reactions were carried out in acid media which cause the fibrous collagen structure to swell, the degree of swelling was regulated by adding sodium sulphate except in one experiment. From Table I, it can be seen that the percent grafting was greater in the absence of sodium sulphate.

Hydroxyproline in grafted collagen

Table I also gives the estimation of total nitrogen and also hydroxyproline and arginine. From the table, it can be seen that the collagen content obtained by the total nitrogen, arginine and weight difference methods agreed within reasonable limits. But the hydroxyproline determination invariably gave lower values. Similar results were also obtained in the case of ceric ion initiated grafting.[1] In the grafting of vinyl monomers onto collagen, the grafts are attached to certain amino acids of the collagen trunk polymer through covalent linkages which are resistant to hydrolysis. When the grafted product is, therefore, hydrolysed with hydrochloric acid, the amino acid through which the grafting has taken place will still be attached to the grafted branches. The lower values obtained for hydroxyproline in the hydrolysates of the grafted collagen, therefore, suggest that some of the grafts may be attached to hydroxyproline residues. However, the

number of grafting sites (vide Table-3) calculated for the various grafted polymers are too few to account for the significant decrease in hydroxyproline content. It is possible that some of the hydroxyproline residues may be modified by attachment with shorter grafts which cannot be isolated by acid hydrolysis. Such a possibility is being examined and the findings will be reported in future communications. It is also possible that some of the hydroxyproline residues in collagen are destroyed by Mn^{3+} ions. However control experiments showed that such losses of hydroxyproline are not very significant.

Rate of grafting and efficiency of grafting

The rate of grafting followed the order MMA> MA> AN; however, it was greater in the absence of neutral salt (Table 2).

TABLE 2

GRAFTING OF VINYL MONOMERS ONTO COLLAGEN[a]

Monomer	*Total yield (g.)*	*Total conversion (%)*	*Weight of homopolymer (g.)*	*Weight of grafted polymer (g.)*	*Efficiency of grafting (%)*
MMA[b]	19.18	57.00	0.004	11.40	100
MMA	20.70	56.35	0.482	11.27	95.86
MA	15.20	42.96	0.209	8.59	97.63
AN	30.35	51.70	0.090	10.34	99.15

a : as in Table 1

b : In the absence of Na_2SO_4

The efficiency of grafting was 95% and above using Mn^{3+} ion initiation. The amount of homopolymer as determined by precipitation of the polymer extracted from the grafted product was found to be less than 5%. It would appear therefore that most of the polymers formed during copolymerisation are grafted to the collagen backbone. Thus the efficiency of grafting is very high using Mn^{3+} initiation technique with different monomers although the rate of grafting varies, depending on the type of monomer used (Table 2). It is possible that the rate of disproportionation of Mn^{3+}—collagen complex may also influence the grafting rate.

Molecular weight of grafted branches

Characterization of different graft copolymers prepared by the manganic ion method showed that the molecular weight of the grafted vinyl polymer is very much larger than that of the collagen substrate indicating that grafting reactions involved only a small proportion of the fibre molecules (Table 3). The fact that the graft copolymers have only a small number of branches is fairly common when they are prepared by a free radical mechanism. Heterogeneity of the grafting system during polymerisation is considered to lead to more structural order in the graft copolymers. The results

TABLE 3

CHARACTERIZATION OF COLLAGEN—VINYL GRAFT COPOLYMERS[a]

Nature of graft copolymer	*$[\eta]$ of isolated grafts*	*Mol. wt. of isolated grafts $M \times 10^{-5}$*	*No. of grafting sites, mole/mole.*	*Grafting rate %/hr.*	*$Rp \times 10^4$ mole/l/sec.*
Collagen-PMMA[b]	4.2	14.55	0.3025	48.9	6.787
Collagen-PMMA	5	18.32	0.2459	39.8	5.524
Collagen-PMA	1.1	2.897	0.5526	17.79	2.873
Collagen-PAN	5	5.623	0.2768	17.29	4.526

a. as in the Table I

b. In the absence of Na_2SO_4

obtained in the case of ceric ion also indicated a similar trend.[5] Molecular weights of PMA and PAN gave lower values compared with PMMA.

In previous investigations[28,30] involving grafting with different initiators, it was found that the molecular weight of the grafted branches is the same as that of the homopolymer! In our investigations with samples obtained under different conditions, the molecular weight of the grafted branches is found to be considerably higher than that of the homopolymer obtained during polymerisation. Thus in the case of PMMA, the grafted side chains had a molecular weight of 18.32×10^5 whereas the corresponding homopolymer had only a molecular weight of 3.12×10^5. These results are in agreement with those obtained with cellulose-PMMA graft copolymers using Mn^{3+} initiation technique.[23]

Grafting sites

The molecular weight of the isolated grafts indicates that the grafted chains are many times larger than the tropocollagen molecules (M. wt. 300,000) making up the collagen fibres (Table 3). The number of grafting sites obtained in most cases indicates that only one collagen molecule in four is involved in the grafting reaction. However, in the case of collagen-PMA grafts, the isolated grafts have lower values of molecular weight and the number of grafting sites was more as compared to MMA grafted samples. The analytical results indicated that hydroxyproline residues may be involved as grafting sites using Mn^{3+} initiation technique. The peptide backbone may also provide sites for initiation of the grafting reaction.

Infra-red spectra

The infra-red spectra of graft copolymers show both the characteristic absorption bands of the polymer grafted and those of the collagen substrate. However, many workers have reported[21,31] that when the infra-red spectra of the graft copolymers were compared with synthetic blends of the same composition, no significant differences could be detected between the spectra of grafts and blends. A better method for establishing proof of grafting was achieved

in the present study by comparing the infra-red spectra of polymers isolated from collagen-vinyl graft copolymers by acid and enzymatic hydrolysis with those of their corresponding homopolymers. Where the products are true graft copolymers, the infra-red spectra of the isolated material will show typical bands of amino acid residues and those of the grafted polymer. In the case of physical mixtures, the isolated product will register the corresponding homopolymer only, since collagen trunk will be removed completely by hydrolysis. The infra-red spectra of a number of grafted side chains isolated from different collagen-vinyl graft copolymers by both acid and enzymatic hydrolysis were therefore examined. As a representative sample, the infra-red spectra of the isolated poly (MMA) side chains obtained by enzymatic and acid hydrolysis is shown in Fig. 1.

The infra-red spectrum of isolated grafts of collagen-MMA graft copolymers by both acid and enzyme (pronase) methods showed characteristic amide absorption bands of collagen at 1660 and 3390 cm^{-1} in addition to absorption bands at 1730, 1450, 1150 and 990 cm^{-1} of PMMA. The spectra of isolated grafts from collagen-PAN and collagen-PMA graft copolymers also showed characteristic amide absorption bands at 1660 and 3390 cm^{-1}, as well as bands of the corresponding homopolymers. The amide absorption bands are more prominent following enzymatic digestion than with acid hydrolysis. These results are in agreement with those obtained using ceric ion initiation[4] since proteolytic enzymes will give rise to longer fragments attached to the end of the grafted side chains, thereby giving prominent peptide amide absorption bands in these cases.

In Table 4, the results obtained with Mn^{3+} initiation technique are compared with those using Ce^{4+} initiation technique as reported in earlier studies.[1-3] It can be seen that in both cases, the molecular weight of grafted branches is very high and only a small proportion of collagen molecules is involved in grafting. Homopolymer formation is also negligible in both cases since free radicals are formed directly on the collagen backbone itself.

TABLE 4

Comparative data of Collagen-PMMA grafts prepared by Ce^{4+} and Mn^{3+} initiation methods

Method	*Percent grafting*[a]	*Grafting rate %/hr.*	$R_p \times 10^4$ *mole/l./sec.*	*Mol. wt. of isolated grafts* $M \times 10^{-5}$	*No. of grafting sites, mole/mole*
Ceric ion method	159.6	53.2	7.384	20.75	0.2301
Manganic ion method	146.7[b]	48.9	6.787	14.55	0.3025
,, ,, ,,	119.3	39.8	5.524	18.32	0.2459

a. Calculated on the basis of nitrogen values.
d. In the absence of Na_2SO_4

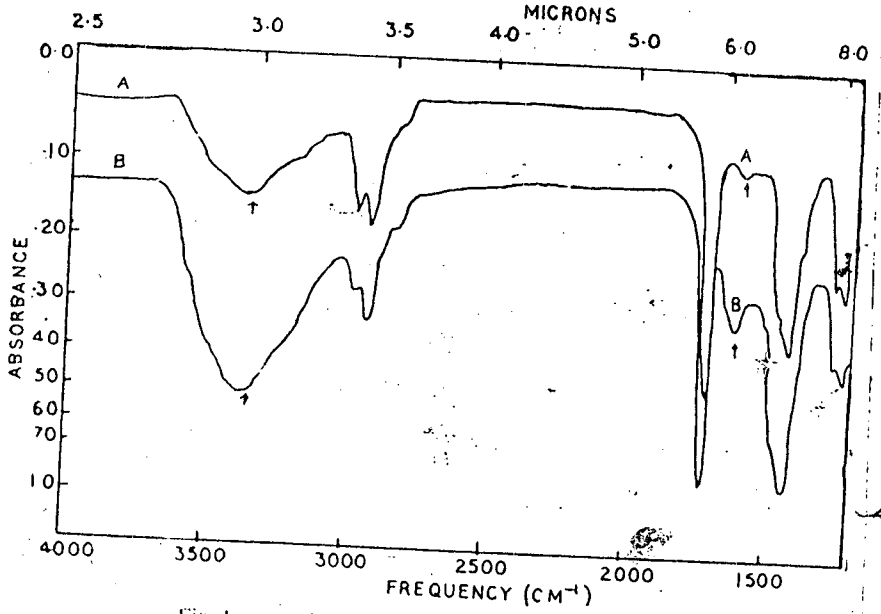


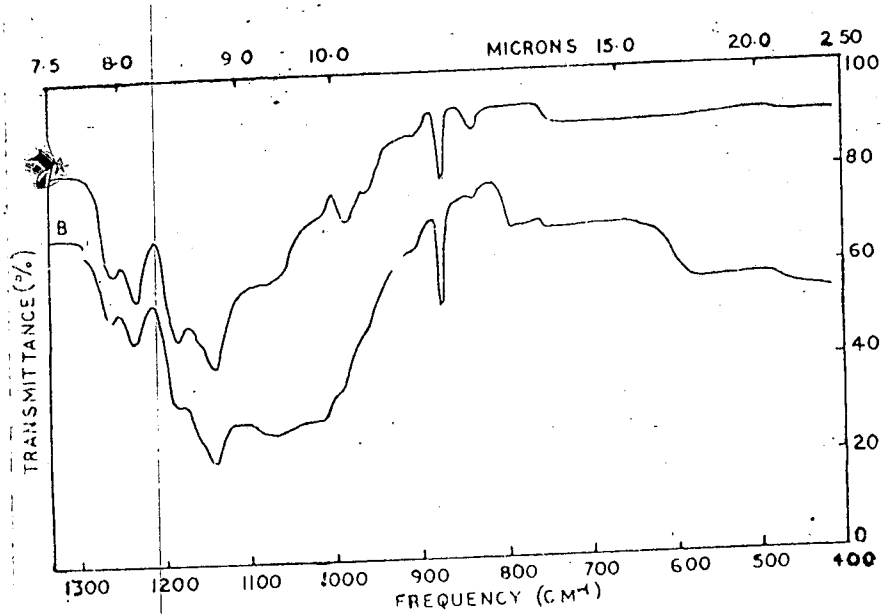
Fig. 1 :— Infra-red spectra of PMMA grafts isolated from collagen—

Electron microscopy

The structure of collagen-vinyl graft copolymers depends on the method of initiation of free radical formation, experimental conditions during polymerisation, pre-treatment of the backbone substrate and the type of monomer used. In the present study, the location and distribution of the grafted polymers in the collagen fibres were studied using electron microscopy, taking the collagen-poly (methyl methacrylate) graft copolymer as a typical sample. Electron microscopic observations of grafted collagen fibrils using manganic ion initiation technique show (Fig. 2) that the fibrils are coated with the polymers resulting in obscuration of the cross-striations. These results are similar to those obtained with ceric ion initiated PMMA graft copolymers which indicated that the grafted polymers penetrate deeply into the fibrils, so masking the cross-striations.

Mechanism of grafting reaction

The most important feature of the manganic ion system is that it proceeds via a single electron tranfer with the formation of free radicals on the reducing agents.[32,33] Thus, if the reducing agent is a polymeric molecule such as collagen and the oxidation is carried out in the presence of a vinyl monomer, the free radical produced on the collagen molecule initiates polymerisation and the production of a graft copolymer. As in other systems such as cellulose and polyvinyl alcohol where grafting is accomplished using free radicals, the present process involves formation of radical sites on the collagen backbone. In a system such as manganic sulphate, methyl methacrylate (or any vinyl monomer) and collagen, the graft copolymerisation reaction is thought to proceed according to the following mechanism. The elementary reactions



PMMA graft-copolymer (A) by acid hydrolysis (B) by Pronase digestion.

(1)-- (6) were assumed for the graft copolymerisation.

Initiation

$$Mn^{3+} + CXH \underset{}{\overset{K}{\rightleftharpoons}} \text{Complex} \xrightarrow{K_d} \dot{CX} + Mn^{2+} + H^+ \quad (1)$$

$$\dot{CX} + M \xrightarrow{K_i} C\,X\,\dot{M} \quad (2)$$

$$Mn^{3+} + M \xrightarrow{K'_i} \dot{M} + Mn^{2+} + H^+ \quad (3)$$

Propagation

$$C\,X\,\dot{M}_{x-1} + M \xrightarrow{K_p} C\,X\,\dot{M}_x \quad (4)$$

$$\dot{M}_{y-1} + M \xrightarrow{K'_p} \dot{M}_y \quad (4')$$

Termination

$$C\dot{X}\,M_x + Mn^{3+} \xrightarrow{K_t} CX\,M_x + Mn^{2+} + H^+ \quad (5)$$

$$\dot{M}_y + Mn^{3+} \xrightarrow{K'_t} M_y + Mn^{2+} + H^+ \quad (5')$$

$$C\dot{X} + Mn^{3+} \xrightarrow{K_o} \text{Oxidative products} + Mn^{2+} + H^+ \quad (6)$$

Fig. 2 :— Electron micrograph of collagen fibrils from hide powder grafted with 119% PMMA, shadowed with palladium-gold. 42,000X

Where C is collagen backbone and X denotes a reactive group in collagen (X = OH, NH_2, CONH etc).

M is a monomer, K is the equilibrium constant and K_i, K'_i, K_p, K'_p, K_t, K'_t, and K_o are rate constants.

Equations (4') and (5') are respectively propagation and termination reactions for homopolymer formation and equation (6) represents the oxidation reaction of collagen radical by the manganic ion.

Since negligible quantities of homopolymer are formed during the graft copolymerisation of collagen with different vinyl monomers under the experimental conditions studied, the reactions (4') and (5') probably occur only to a minor extent. The termination inside the collagen molecule by mutual interaction of the growing graft polymer radicals is unlikely as may be seen by considering the molecular weight data. However, more detailed studies on the kinetics of graft copolymer of collagen, vinyl monomer and manganic ion system are needed to draw any definite conclusions on the nature of the termination step.

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