# Biodegradation of Gelatin Graft Copolymers. III

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## Synopsis

Gelatin-*g*-poly(methyl acrylate) and gelatin-*g*-poly(acrylonitrile) copolymers were prepared in an aqueous medium using $K_2S_2O_8$ initiator. A plausible mechanism has been put forward for the observed grafting behavior of monomers. Gelatin-*g*-PAN showed a greater resistance to mixed bacterial inolucum compared to gelatin-*g*-PMA samples. The rate of degradation decreased with the increase in grafting efficiency. A parallel set of experiments carried out by employing the samples as the only source of both carbon and nitrogen showed a marginal but definite increase in the utilization of the polymer. The nitrogen analysis also showed the utilization of the polymer. Scanning electron micographs of the polymer films do show extensive pitting after microbiological testing.

## INTRODUCTION

For many biomedical, agricultural, and ecological uses, it is preferable to have a biodegradable polymer that will undergo degradation in the physiological environment or by the microbial action in the soil.[1,2] Many natural polymers, although biodegradable, decompose on heating before they melt. On the other hand, most of the synthetic polymers are resistant to enzymatic attack.[3] Modification of the natural polymers is one of the outlined approaches to develop biodegradable systems with desired mechanical behavior.

Gelatin has found many applications in biomedical field as a bioabsorbable agent. For surgical use, gelatin is prepared as a foam or film and crosslinked with formaldehyde to decrease its solubility. These materials have little wet strength and find utility primarily in hemostasis and in the filling of voids after tissue resection.[4] A new surgical procedure was developed using bioabsorbable crosslinked gelatin cup prosthesis which participates in the temporary substitution of tissues.[5] Formaldehyde-crosslinked gelatin also has been used as a matrix in controlled drug release applications for the release of highly water-soluble hydrocortisone acetate.[6]

Another potential approach to modify natural polymers is by grafting. In recent years, there have been some reports on the modification of gelatin by graft copolymerization.[7-10] Further chemical modification of gelatin can bring about improvements in the overall usefulness of this important protein. Keeping in view the prospects for biodegradable polymers especially in nonmedical areas and the well-known ability of gelatin to biodegrade,

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we have reported earlier the biodegradation of some gelatin graft copolymers.[11-15] The present series of papers deal with the biodegradation behavior of gelatin-*g*-poly(methyl acrylate) and gelatin-*g*-polyacrylonitrile using single and mixed bacterial inoculum.

## EXPERIMENTAL

### Materials

Gelatin, bacteriological (BDH) was used in the present investigation. The methods, described by Riddick and Bunger,[16] were adopted for the purification of monomers. $K_2S_2O_8$ was used as the free radical initiator. The bacterial cultures were obtained from the culture collections of the Cell Biology and Microbiology Laboratory, Indian Institute of Science, Bangalore, and routinely maintained on nutrient agar slants.

### Preparation of Gelatin Graft Copolymers

All the grafting experiments were carried out in a three-necked round-bottom flask fitted with a glass stirrer, a gas inlet, and a water condenser. In a typical experiment, gelatin (10 g) was added to the solution, and grafting sites were initiated on the protein backbone by the addition of 0.01 mol of $K_2S_2O_8$. All the grafting experiments were carried out at 60°C and 70°C with constant stirring. The product was purified as reported earlier.[12,13] The occluded homopolymer chains of poly(methyl acrylate) and polyacrylonitrile were extracted with acetone and dimethylformamide, respectively.

### Analysis and Characterization of Graft Copolymers

Grafted side chains were removed from the protein backbone and percent grafting; the efficiency of grafting and the number of grafting sites were calculated as reported earlier.[11] The grafted samples were analyzed for total nitrogen content by Kjeldahl's method. The IR spectra were recorded with Carl-Zeiss Model Sp 700 Infrared Spectrophotometer using KBr pellet.

### Film Preparation

The dry polymer was hot pressed into films of uniform thickness (2 mm) at a pressure of 100 kg/cm$^2$. The die was maintained at a temperature of 140–150°C. However, the melt flow of the samples was very low.

### Microbiological Testing

For testing biodegradability, the mineral salts medium with the polymer in question as the sole source of carbon was used, and the testing was carried out according to ASTM guidelines.[17] A parallel set of experiments were also carried out by using the polymer as the only source of carbon and nitrogen by deleting $NH_4NO_3$ from the above medium.

The growth of bacteria, i.e., *Pseudomonas aeruginosa, Bacillus subtilis,* and *Serratia marcescens,* was followed by recording absorbance of the culture medium every 24 h in a Bausch and Lomb Spectonic 20 Calorimeter at 600 nm. pH measurements were also carried out on Beckman Digital pH Meter.

Scanning electron micrographs of the polymer surface were recorded on Cambridge 150 Model Stereo Scan and the operating voltage of the instrument was 4 kV. The samples were coated prior to examination.

## RESULTS AND DISCUSSION

The grafting experiments were carried out at 60 and 70°C for different intervals of time. Kuwajima et al.[9] reported the $K_2S_2O_8$ is the most efficient radical initator for the graft copolymerization of MMA onto gelatin. Kumaraswamy et al.[8] reported that persulfates required elevated temperatures for the effective grafting to take place. The chosen experimental conditons were sufficient enough to keep the gelatin completely dissolved and to allow the initiator to decompose completely into radicals.

Gelatin–methyl acrylate copolymers showed absorption bands of amide carbonyl group (1660 $cm^{-1}$) due to peptide linkages of gelatin and at 1725 $cm^{-1}$ due to ester carbonyl group of polyacrylic moiety, indicating that grafting of synthetic polymers has been achieved. Aspartic and glutamic acids also contain carboxyl groups. However, the characteristic strong absorption band at 1725 $cm^{-1}$ cannot be attributed to these carboxyl groups because of their low relative concentration in the protein and more so in the case of grafted samples. In the case of gelatin-*g*-polyacrylonitrile, the presence of both 1660 $cm^{-1}$ peak and a peak at 2240 $cm^{-1}$ due to cyano group was taken as the evidence for grafting. Moreover, the grafted chains isolated by the acid hydrolysis, did not show the prominent peak at 1660 $cm^{-1}$ due to peptide linkages. This might be due to the fact the backbone protein was completely hydrolyzed, and the high molecular weight polymeric branches isolated might contain an insignificant number of amino acid or peptide residues.

Table I shows that the yield of the graft copolymers reached a plateau after an hour at 60 and 70°C. However, it must be pointed that in all the cases, after an hour, the insoluble graft copolymer got completely precipitated out and the reaction reached a stage where it could be considered as complete. At 60°C, the efficiency of grafting and the percent grafting were lower in the early stages of polymerization. Significant increase both in the efficiency and the percent grafting was observed with progress of

TABLE I
Preparation of Gelatin–Methyl Acrylate Graft Copolymers

| Sample[a] | Graft copolymer (%) | Free gelatin (%) | Homopolymer of poly(methyl acrylate) (%) |
|---|---|---|---|
| At 60°C | | | |
| 30 min | 19.5 | 49.0 | 31.5 |
| 60 min | 58.0 | 30.0 | 12.0 |
| 120 min | 59.0 | 31.0 | 10.0 |
| At 70°C | | | |
| 30 min | 35.0 | 55.6 | 9.4 |
| 60 min | 60.0 | 25.0 | 15.0 |
| 120 min | 62.6 | 25.4 | 12.0 |

[a] In each case, 10 g of gelatin, 0.54 g $K_2S_2O_8$, and 10 mL of methyl acrylate were added to 200 mL of water.

polymerization (Table II). In other words, the homopolymer formation was greater in the early stages of polymerization (30 min), and thereafter there was much less homopolymer formation. The molecular weights of the grafted branches at 60 min of polymerizations at 60°C were low, but there was a corresponding increase in the grafting sites.

At 70°C, at shorter time of polymerization (30 min), the efficiency of grafting, the number of grafting sites, and the percent conversion were relatively high. However, the molecular weight of the grafted branches was low. Prolonged time of polymerization (60 and 120 min) resulted in the increase in the percent conversion and graft yield considerably.

Due to the difficulties involved in the analysis, only the sample of gelatin-*g*-polyacrylonitrile was prepared at 70°C using 0.01 mol of $K_2S_2O_8$ initiator. The percentage of protein in the grafted sample, as determined by acid hydrolysis, was 50% by weight ($\overline{M}_v \simeq 30{,}000$).

Quantitatively, three salient features are noticeable from these results. First, the number of grafted branches per gelatin molecule is small, and their molecular weights are high. This could be explained by the fact that the polymerization system was heterogenous, and, when a hydrophobic synthetic polymer chain starts growing on gelatin backbone, the latter become insoluble, though it remains swelled. Hence the probability for the free radicals generated in the aqueous phase from water-soluble $K_2S_2O_8$ for mutual termination becomes high and prefer to terminate each other. The free radicals, therefore, undergo very little termination with the growing chain, resulting in high molecular weights of the grafted branches. The small number of side branches may be attributed to the structure of gelatin, where the number of —CH(OH) groups responsible for the generation of free radicals on the gelatin backbone is small in number[18] (Table III). Secondly, the time of polymerization has an important effect. Lastly, from the practical view point, the temperature effect on the polymerization is highly interesting.

In order to explain these results, a mechanism similar to that put forward earlier by Kumaraswamy et al.[8] in the case of grafting of MMA onto gelatin could be invoked. The mechanism proposed is given below:

$$I_2(S_2O_8^{-2}) \rightarrow 2I\cdot(SO_4^{\cdot}) \tag{1}$$

*Direct grafting*:

$$I\cdot + \text{Gel—H} \rightarrow \text{I—H} + \text{Gel}\cdot \tag{2}$$

$$\text{Gel}\cdot + n\text{M} \rightarrow \text{Gel—(M)}_{n}\cdot \tag{3}$$

*Grafting by chain transfer*:

$$I\cdot + \text{M} \rightarrow \text{IM}\cdot \xrightarrow{\text{M}} \text{I} - (\text{M})_{m}\cdot \tag{4}$$

$$\text{Gel—H} + \text{I—(M)}_{m}\cdot \rightarrow \text{Gel}\cdot + \text{I—(M)}_{m}\text{—H} \tag{5}$$

TABLE II
Composition of Gelatin–Methyl Acrylate Graft Copolymers

| Sample | Nitrogen (%) | Gelatin (%) | Poly(ethyl acrylate) (%) | Grafting (%) | Efficiency of grating (%) | Conversion (%) | $\bar{M}_n$ of poly(methyl acrylate) $\times 10^{-5}$ | No. of grating sites (mol/mol) |
|---|---|---|---|---|---|---|---|---|
| At 60°C | | | | | | | | |
| 30 min | 8.51 | 51.6 | 48.4 | 93.8 | 23.05 | 53.2 | 8.0 | 0.155 |
| 60 min | 5.79 | 35.1 | 64.9 | 18.4 | 75.8 | 64.5 | 6.5 | 0.255 |
| 120 min | 5.52 | 33.5 | 66.5 | 199.2 | 79.8 | 62.9 | 6.5 | 0.2758 |
| At 70°C | | | | | | | | |
| 30 min | 5.20 | 31.51 | 68.59 | 185.0 | 67.0 | 139.5 | 4.2 | 0.3423 |
| 60 min | 6.8 | 41.21 | 58.79 | 143.05 | 70.18 | 117.4 | 6.8 | 0.1893 |
| 120 min | 6.6 | 40.60 | 59.40 | 146.3 | 75.6 | | 6.8 | 0.1936 |
| 106.06 | | | | | | | | |

[a] In each case 10 g of gelatin, 0.54 g $K_2S_2O_8$, and 10 mL of methyl acrylate monomer were added to 200 mL water.

TABLE III
Functionally Active Groups in Gelatin

| Nature of functional group | No. of functional groups (per 60,000 mol wt) |
|---|---|
| α-Amino- | 1 |
| ϵ-Amino- | 20 |
| Guanidino- | 30 |
| Carboxyl- | 50–78 |
| Hydroxyl-[a] | 105 |
| Peptide bonds | 657 |

[a] Gelatin contains —OH groups on serine, threonine, hydroxyproline, and hydroxylysine residues.

*Termination*:

$$\text{Gel}\cdot + \cdot(\text{M})_m\text{—I} \rightarrow \text{Gel—(M)}_m\text{—I} \quad (6)$$

where G = gelatin, M = monomer, and I = initiator.

The results indicate that, in the presence of $K_2S_2O_8$, the grafting occurs by chain transfer at 60°C. It is well known that, in chain transfer, numerous radicals are generated, some of which do not contribute towards grafting. The low efficiency of the grafting at 60°C, 30 min, could be due to ineffectiveness of the primary radicals to produce radical sites directly on the protein backbone in the early stages and because the reaction was mainly due to chain transfer by macroradical (step 5). It can also explain the simultaneous formation of more homopolymer at lower temperature and short polymerization time.

One of the possible interpretations for the increase in the grafting efficiency (at 60°C, 60 min and 70°C, 20 min) could be the combined effect of both primary radicals ($SO_4^{\cdot}$) and growing macroradicals of syntheti moiety (steps 2 and 5) in producing active sites on the protein backbone by chain transfer. This possibility was visualized by workers in grafting poly(vinyl alcohol) using $K_2S_2O_8$.[19] Similar results were obtained by Hayashi et al. while studying the graft copolymerization of MMA onto gelatin.[8]

The increase in the efficiency of grafting, accompanied by an increase in the number of grafting sites and decrease in their molecular weights, can also be explained in the following way. The primary radical may attack the PMA chain, as well as the gelatin backbone at higher temperatures, resulting in the lower molecular weights of the grafted branches. In addition, macroradicals are expected to recombine with the backbone radicals in the latter periods of polymerization, when the homopolymerization cannot proceed because of absence of monomer. Therefore, the conversion from homopolymer to grafted branches results in an increase in the efficiency of grafting.

Three samples of these graft copolymers with grafting efficiencies of 23.05% (8.51% N), 70.18% (6.8% N), and 79.80% (5.52% N), designated as GMA, GMB, and GMC, respectively, were selected for testing the bacterial susceptibility. As discussed earlier, the selection of an appropriate organism in performing a biodegradability test is dependent upon the objectives of

the experiment. Test organisms in the present investigation are *Pseudomonas aeruginosa, Bacillus subtilis*, and *Serratia marcescens.*

As in all our previous experiments, we have selected the percent weight loss of the samples as the objective criterion to follow degradation. All the tubes were inoculated in triplicate. To ensure that estimates of behavior are not too optimistic, the greatest degree of deterioration is reported which has been the convention.[17] The percent weight loss vs. time plots are shown in Figures 1 and 2 for gelatin-*g*-PMA and gelatin-*g*-PAN systems respectively, incubated with the mixed bacterial inoculum. Figure 1 shows that the extent of degradation is in the following order:

$$\text{GMA} > \text{GMB} > \text{GMC}$$

It is also to note the greater resistance of gelatin-*g*-PAN to microbial attack (Fig. 2), compared to gelatin–methyl acrylate or gelatin–ethyl acrylate systems reported earlier.[11,12] The gelatin-*g*-PAN having equivalent amount of protein exhibits a lower percent weight loss. The present study suggests the possibility of the effect of the nature of grafted branches on the degradation of the backbone. It can be explained on the basis of enzyme-inhibitory effect of cyano groups of PAN moiety. Similar resistance to enzymatic oxidation was reported in the case of butyl and nitrile rubbers,

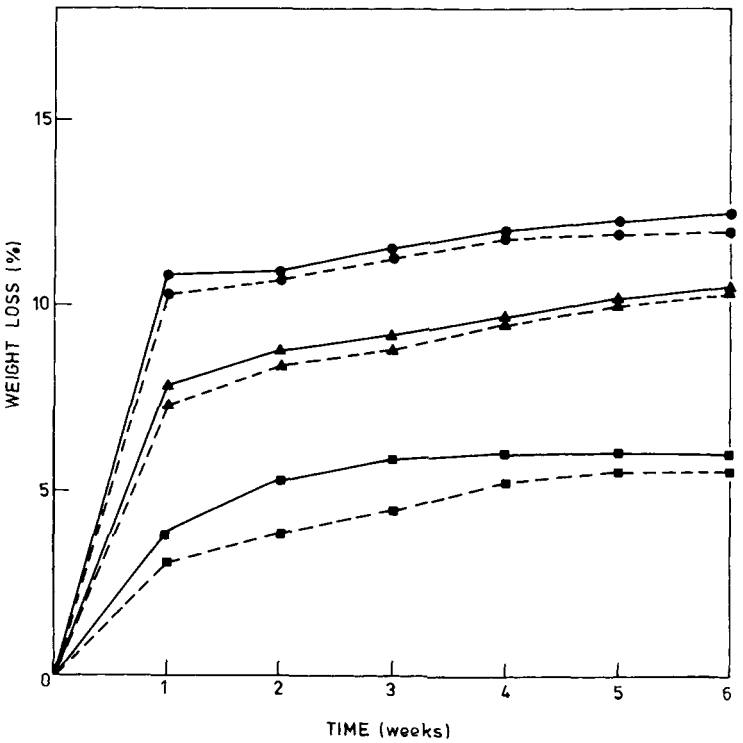


Fig. 1. Weight loss vs. time curves of gelatin-*g*-Poly(methyl acrylate) samples incubated with *Pseudomonas Aeruginosa, Bacillus subtilis,* and *Serratia marcescens* in nitrogen-free (—) and nitrogen-rich (- - -) media: (●) GMA; (▲) GMB; (■) GMC.

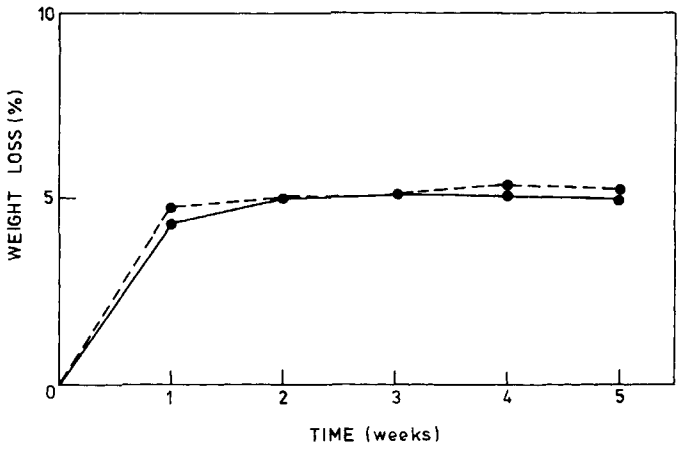


Fig. 2. Weight loss–time curves of gelatin-*g*-poly(acrylo nitrile) (50% of pan by weight) incubated with *Pseudomonas aeruginosa, Bacillus subtilis,* and *Serratia marcescens* in nitrogen-rich (- - -) and nitrogen-free (—) media.

where the nitrile rubber was found to be completely resistant to microbial attack.[20] It was explained that the cyano group in nitrile rubber might have conferred some bacteriocidal properties. Further increase in the efficiency of grafting of PAN onto gelatin may result in the total resistance of these polymers to microbial action. Hence, these graft compositions may be considered for glaze finishing on leathers where the microbial resistance is a desirable factor.

Further evidence for the degradation of the polymer was obtained from the estimation of nitrogen content of the samples before and after microbiological testing (Table IV). The results indicate positively the utilization of the gelatin portion of the molecule.

The growth of bacteria, monitored by reading the absorbance at 660 nm, showed a sharp increase during the first week (Fig. 3). The lag period, being less in nitrogen-free medium compared to nitrogen-rich medium, indicates a better attack on the polymer in the absence of added nitrogen source.

The scanning electron micrographs of gelatin-*g*-poly(methyl acrylate) films after and before testing for the bacterial susceptibility are shown in Figure 4. The incubated samples show extensive pitting and erosion of the material. The pitting, channeling or cavernous appearance resulting from

TABLE IV
Total Nitrogen Content of Gelatin–Methyl Acrylate Copolymers Inoculate with *Pseudomonas Aeruginosa, Bacillus Subtilis,* and *Serratia Marcescens*—before and after Testing

| Sample | Initial nitrogen (%) | After 15 days (nitrogen %) | | After 45 days (nitrogen %) | |
|---|---|---|---|---|---|
| | | N(+) medium | N(−) medium | N(+) medium | N(−) medium |
| GMA | 8.51 | 5.85 | 5.6 | 4.28 | 3.85 |
| GMB | 6.8 | 4.78 | 5.0 | 3.80 | 4.0 |
| GMC | 5.52 | 3.9 | 4.1 | 3.3 | 3.52 |

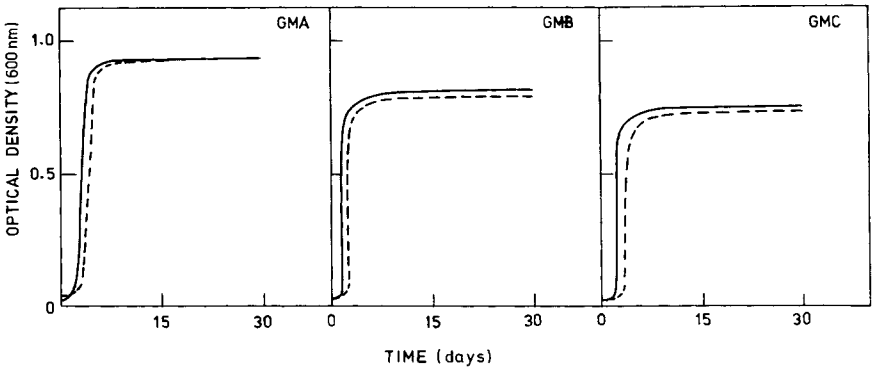


Fig. 3. Optical density of the culture medium at 600 nm vs. time plots of gelatin-*g*-poly(methyl acrylate) samples incubated with *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* in nitrogen-rich (- - -) and nitrogen-free (—) media.

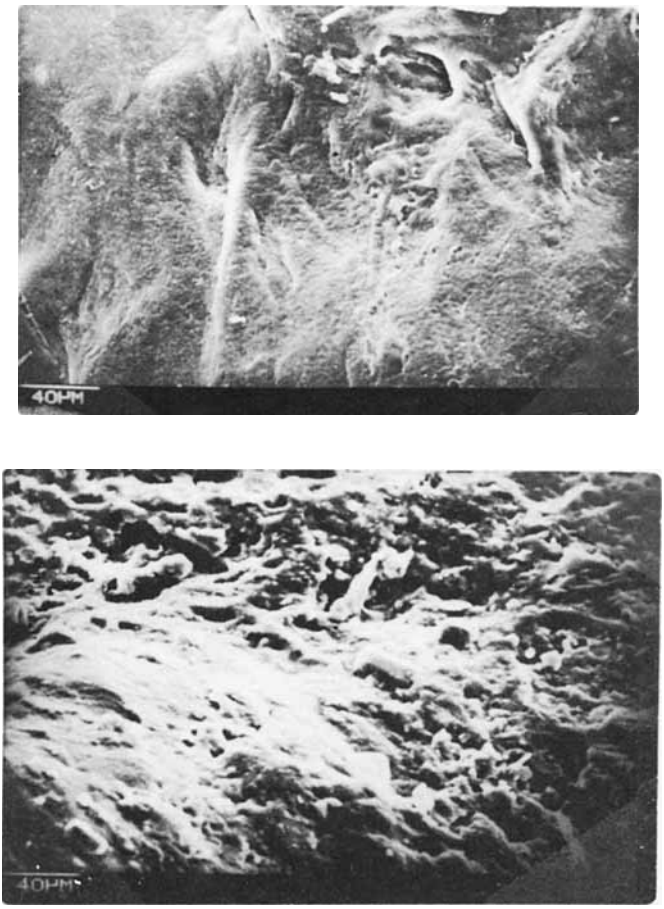


Fig. 4. Scanning electron micrographs of gelatin-*g*-PMA before (above) and after (below) incubation with mixed cacterial inoculum.

the degradation process is readily apparent. Presumably, these pits are caused by the utilization of the protein portion of the copolymer as a carbon source by the microorganisms.

The observed phenomenon of rapid weight loss during the first week and the concurrent cooperative changes such as exponential increase in bacterial population indicate early rapid hydrolysis of the backbone followed by a period of nearly uniform rate at an appreciably reduced level. Scanning electron micrographs and total nitrogen estimation of the samples, before and after testing, also confirm biodegradation.

## CONCLUSIONS

The characteristic feature of grafting of methyl acrylate onto gelatin was the small number of PMA branches per gelatin molecule and their high molecular weights. The extent of degradation decreased with the increase in the efficiency of grafting. There was an initial rapid utilization of the polymer by the bacteria followed by slow but steady degradation. Gelatin-*g*-polyacrylonitrile was found to be more resistant to mixed bacterial attack. When the polymer samples were used as the only sources of both carbon and nitrogen, there was marginal but definite increase in the utilization of the gelatin–PMA samples.

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