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Azo Crosslinkers, Synthesis and Their Reduction

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Polymers containing azo crosslinks have been used as coating devices and protect the drug in a solid dosage form. They are reduced by colonic bacteria which allow drug release within the colon. This study was concerned with the investigation of the mechanism of release by bacterial reduction of azo crosslinkers. Chain-growth copolymers containing hydrophilic and hydrophobic monomers and a bifunctional azo aromatic crosslinker were prepared and their reduction in bacterial cultures was investigated. The polymers swelled and their dry weight decreased on incubation in aqueous media. The amount of weight loss increased with increasing proportion of the hydrophilic monomer. There was no enhancement of weight reduction in bacterial cultures which had demonstrable azo reduction activity, suggesting that cleavage of azo bonds in the polymer network did not take place.

Key Words: Azo polymers, Bacteria reduction, 2-Hydroxyethyl methacrylate, Styrene, Drug delivery.

INTRODUCTION

The effectiveness of low molecular weight azo compounds in delivering drugs to the colon has lead to the development of new approaches to colonic drug delivery which rely on the reductive cleavage of azo bonds by the colonic microflora as the trigger mechanism for drug release. The polymer is water-impermeable and not susceptible to gastrointestinal digestive enzymes. On arrival of orally administered coated capsule at the ileocaecal junction, the azo bonds in the coating would be cleaved by the colonic bacteria, disrupt the polymer network and make it permeable to fluids and drugs. Hydrophilic polymer networks (hydrogels) derived from polymers or copolymers of methacrylic esters, such as 2-hydroxyethyl methacrylate (HEMA) as a co-monomer and azo aromatic act as a crosslinker between the polymer chains may be used for colon-specific drug delivery. The polymers have been shown to be well tolerated by biological systems and have many biomedical applications. 2-Hydroxyethyl methacrylate is commonly polymerized by free radical chain-reaction polymerization in which an

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initiator, such as benzoyl peroxide, reacts with a carbon-carbon double bond to yield a reactive intermediate, a carbon radical. The polymer is built as more monomers add successively to the reactive end of the growing chain. The active site can be any of a wide range of substituents, such as –H, alkyl groups, various functional groups or halogens. Inclusion of monomers containing two vinyl groups in such polymerization process can lead to crosslinking and network formation.

Hild and Rempp[1] carried out a kinetic investigation of copolymerization of several systems of HEMA, such as styrene-divinylbenzene and methylmethacrylate-ethylene dimethacrylate. They concluded that the bifunctional monomers were more reactive and therefore, more readily consumed than the monofunctional monomers. They suggested, in the early stages of the reaction, that most of the bifunctional monomers react to yield pendant double bonds. The reactivity of these bonds is far lower than that of the monomers involved. As these double bonds are consumed in the later stages of the reaction, when not much monomer is left over, the network becomes tighter and swelling ratio of the polymer decreases. This generates a more or less homogeneous spread of crosslinker within the polymer network and the crosslink density can be related to the composition of the monomer mixture, provided the reaction was pursued until all double bonds had been consumed. Saffran *et al.*[2] have suggested that polymers of HEMA are insoluble in water but, due to the presence of an alcohol group in the HEMA monomer, they have limited compatibility with water and swell in aqueous media to allow the diffusion of aqueous solutions within the polymer.

Polymerization of unsaturated monomers is complicated in practice by several factors that greatly affect the properties of the product. One such problem is; radical polymerization often yields a product that is not linear, but has numerous branches[3]. Various molar ratios of crosslinker: other monomers have been used, *e.g.* 1:250[4] and 1:200[2], but capsule coatings made of such polymers showed imperfections and allowed permeation of water when they were immersed in aqueous media[5]. Crosslinking of polymers tends to increase structural rigidity, because the individual chains can no longer slip over each other but instead locked together into immense single molecules[3]. Polymers may be preserved from becoming brittle by the addition of plasticizers, small organic molecules that act as lubricants between polymer chains. However, plasticizers are generally toxic and as such unsuitable for use in oral pharmaceutical preparations. The problem of high rigidity and low flexibility of crosslinked polymers may possibly be reduced by using longer crosslinker molecules, which offer more freedom of movement about the crosslinks[6].

Pradny and Kopecek[7] suggested that susceptibility of an azopolymer to enzymic degradation by microbial azo reductases is dependent on the swellability of the polymer, which is in turn influenced by the relative proportions of the hydrophilic and hydrophobic monomers incorporated in the polymer. Some workers[4,8] have investigated the swellability or the viscosity of the polymers before and after *in vitro* or *in vivo* incubation with caecal contents.

It should be noted that quantitative and qualitative characterization of caecal bacteria flora is a complicated process due to factors such as requirement for strict anaerobic conditions, inter-species variations and the influence of diet on colonic microflora[9]. Spectrophotometric methods have been used to monitor azo reduction in the polymers, which often exhibit the colour of the azo crosslinker[10,11]. Permeability studies on thin films of azo polymers have been used to show that permeability of the films to small molecules increase on incubation in bacterial cultures[12]. However, the results do not prove that microbial azo reduction was responsible for the increased permeability.

Although the polymers of the types of azo crosslinked polyHEMA and those synthesized by previous workers may have provide useful information about azo polymer degradation and coating properties. They should be regarded only as crude and simple prototypes since there has been little control over the monomer sequence, crosslinking, branching and molecular weight of the polymers.

The principle of biodegradable azopolymers is unusual in the sense that the majority of biodegradable polymers are designed to be disintegrated by hydrolysis rather than azo reduction. Although there is extensive information available on biodegradation of polymers by hydrolysis, relatively little is known about biodegradation of azopolymers. The ability of many bacteria and mammalian cells to cleave the azo bonds in low molecular weight azo compounds and water soluble high molecular weight polymeric derivatives of certain azo dyes has been demonstrated[13,14], there is no reliable evidence to suggest that the insoluble azopolymers of the type proposed for colonic drug delivery are biodegradable through azo reduction by biological systems. Some investigators studied drug delivery to the colon that can be achieved in different ways coating with azo crosslinked drugs with bacterially degradable polymers[15-17]. Mahkam *et al.*[18] studied azo polymer containing polyurethanes for colon-specific drug delivery. Tozaki *et al.*[19,20] showed that azo polymer-coated pellets may be useful carriers for the colon-specific delivery of peptides including insulin and budesonide.

The experiments regarding to swallability of azo polymers are unable to distinguish the changes in macromolecular structure which occur to the

polymer as a result of azo reduction which would occur upon degradation of the polymer by some other mechanism, *e.g.*, hydrolysis of hydroxyl ethyl esters. These studies provide useful information relating to the caecal degradation of such polymers but it has not been conclusively demonstrated that degradation occurs as a consequence of azo reduction.

The purpose of this study was to investigate the bacterial reduction of a range of azo polymers with different degrees of hydrophilicity by preparing a range of polymers containing various proportions of HEMA, as a hydrophilic monomer, styrene as a hydrophobic monomer and dimethacryloyloxyazobenzene as a bifunctional crosslinker.

EXPERIMENTAL

Benzoyl peroxide, HEMA and styrene were obtained from Aldrich Chemicals Company Ltd. HEMA and styrene contained polymerization inhibitors, hydroquinone monomethyl ether (300 ppm) and *p*-*t*-butylcatechol (10 ppm), respectively. 2,2´-Dimethacryloxyazo benzene and amaranth were obtained form Ciba Pharmaceuticals and BDH Chemicals Ltd., respectively.

Enterococcus faecalis NCIMB775 was obtained from the National Collection of Industrial and Marine Bacteria and maintained on nutrient agar at 4ºC and subcultured at *ca.* 3 monthly intervals. Caecal bacterial content was obtained from male Wistar outbred rats.

Microbiological media was prepared using nutrient broth, oxoid CM1, thioglycollate broth and phosphate buffered saline (PBS) tablets which were obtained from Unipath Ltd. They were prepared and sterilized according to the manufacturers instructions. The compositions of the media are described in Table-1. The thioglycollate broth also contains sodium thioglycollate, methylene blue and oxoid L11 with the compositions of 1.0, 0.002 and 1.0 (g/L), respectively. Nutrient agar DM179 was obtained from Mast Laboratories Ltd. and was prepared and sterilized according to the manufacturers instructions.

In all microbiological work aseptic techniques were adopted using sterile equipment in a laminar flow cabinet, where appropriate, to avoid contamination of the cultures.

In polymer synthesis, HEMA was distilled under reduced pressure, 3.5 mm Hg and 67ºC to remove the polymerization inhibitor, *p*-*t*-butylcatechol. The inhibitor in styrene was also removed by extraction into an equal volume of 5 % (w/v) aqueous NaOH. Then, a mixture containing 10 mL methanol, 0.050 g 2,2´-dimethacryloxyazo benzene, 0.120 g benzoyl peroxide $(PhCO_2)_2$ and 10 g the monomers (2-10 g HEMA and 0-8 g styrene) in total was placed in a round bottom flask and heated at 80ºC, with stirring, until polymerization was complete. The polymer was precipitated by addition of an excess of water and washed twice with water

and ethanol. It was dissolved in methanol and transferred to a 100 mL conical flask. A small amount of the methanolic solution of each polymer was poured on a siliconised glass slide to form a thin film of about 2 cm diameter and was allowed to dry at 40ºC. Infrared spectra of the dried films were obtained. To prepare cultures of *E. faecalis*, sterile nutrient broth CM1 (100 mL) in a 250 mL conical flask was inoculated with a colony of *E. faecalis* and incubated, without shaking, at 37ºC overnight (for not less than 15 h). This dense bacterial suspension was used to inoculate other cultures.

TABLE-1
COMPOSITIONS OF MICROBIOLOGICAL BROTH

Thioglycollate broth	Lab-lemco powder	Yeast extract	Peptone	Sodium chloride	Dextrose
Composition (g/L)	1.0	2.0	5.0	5.0	5.0
Nutrient broth	Lab-lemco powder	Yeast extract	Peptone	Sodium chloride	–
Composition (g/L)	1.0	2.0	5.0	5.0	–
Phosphate buffered saline	Potassium dihydrogen phosphate	Potassium chloride	Disodium hydrogen phosphate	Sodium chloride	–
Composition (g/L)	0.2	0.2	1.15	8.0	–
Nutrient agar DM179	Peptone A	Yeast extract	Beef extract	Sodium chloride	Agar A
Composition (g/L)	6.0	2.0	1.0	5.0	14.0

TABLE-2
POLYMERIZATION TIMES OF AZO CROSSLINKED CO-POLYMER

HEMA content (g)	10.0	9.5	9.0	8.0	6.0	4.0	2.0
Styrene content (g)	0.0	0.5	1.0	2.0	4.0	6.0	8.0
Polymerization time (h)	0.5	1.0	1.5	2.5	4.0	5.5	6.5

Surface spread method was adopted to count incubation colonies. A sample of the bacterial suspension (1 mL) under investigation was withdrawn and serially diluted with sterile 0.01 M phosphate buffered saline at pH 7.4 to produce an estimated final concentration of between 250 and 1000 cells/mL. A sample (0.2 mL) of each appropriate dilution was pipetted onto a surface dried nutrient agar plate and spread evenly using a sterile glass spreader. The liquid was allowed to be absorbed into the agar before incubating the plates at 37ºC. The colonies developed on the plate after 48 h of incubation were counted and assuming that each colony was formed by one colony forming unit (cfu). The concentration of the bacteria in the original culture was calculated from the mean colony counts of three plates. Samples from uninoculated cultures were used without dilution.

In the reduction of amaranth in cultures of *E. faecalis*, different medium were used. For the reduction in anaerobic thioglycollate medium, amaranth was dissolved in 1 L thioglycollate broth to give a concentration of 0.01 % w/v. The solution was distributed into screw capped universal bottles and sterilized in an autoclave at 121ºC for 15 min. The red colour of the amaranth solution disappeared during the sterilization process, therefore, the use of thioglycollate medium was abandoned and nutrient broth was used instead. For the reduction in nutrient broth, thirty six universal bottles each containing a solution of amaranth (0.01 % w/v) in nutrient broth CM1 (25 mL) were sterilised in an autoclave at 121ºC for 15 min. The solutions were maintained at 37ºC, inoculated with 0.4 mL of an overnight culture of *E. faecalis* and incubated at 37ºC. Six bottles were taken out of the incubator at each time interval and a sample (5 mL) from each bottle was centrifuged at 3000 g for 10 min at 4ºC to remove the bacteria. Absorbance of the supernatant solution was measured spectrophotometrically at the absorbance maximum of amaranth (522 nm) employing nutrient broth as reference. When the absorbance was greater than 1.5, the samples were diluted with a known volume of nutrient broth. In control experiments, the solution of amaranth in nutrient broth was inoculated with 0.4 mL sterile nutrient broth instead of the *E. faecalis* suspension. The concentration of viable bacteria in the cultures was estimated at the end of incubation period using the surface spread method. For the reduction of amaranth by rat caecal contents, male Wistar rats were sacrificed by carbon dioxide inhalation 5 min before the start of the experiment. The rat caecal contents, 4 g, were used to inoculate sterile solutions of amaranth (0.01 % w/v) in nutrient broth, 21 mL, in universal bottles and the suspensions were incubated at 37ºC. In control experiments, the caecal contents were wrapped in aluminum foil and sterilized in an autoclave at 121ºC for 15 min before being added to the solution of amaranth in nutrient broth. Sample collection and absorbance measurements were carried out.

In incubation of polymers in bacterial cultures, uniform-size glass beads, 1 cm in diameter, were weighed and sterilized by soaking in 70 % (v/v) ethanol for 0.5 h. A film of each polymer was coated on a glass bead by dipping the bead in a methanolic solution of the polymer for a few seconds and allowing it to dry to constant weight. The glass beads were designed to maximize the surface area of the polymer exposed to the bacterial culture, by small glass projections which prevented contact between the coated bead and the culture container. Each polymer coated bead was weighed to the nearest 0.1 mg and placed in sterilized nutrient broth, 23 mL, in a universal bottle, inoculated with 0.4 mL of an overnight culture of *E. faecalis* and incubated at 37ºC for 10 d. The bead was then removed from the culture, washed under a gentle stream of distilled water, dried at 60ºC for

24 h and reweighed. Polymer degradation was determined in terms of weight loss during incubation by calculating the changes in the weight of the polymer as a percentage of its original weight. The surface of the polymer was examined under microscope before and after incubation for any signs of change in surface morphology. The experiment was repeated using rat caecal contents, 4 g, instead of *E. faecalis* as the inoculum. Controls were carried out by placing polymer coated beads in a) culture medium, inoculated with 0.4 mL the sterile medium b) sterilized rat caecal contents in nutrient broth and c) deionized water.

In the examination of effects of shaking and acid-alkaline media on polymers, polymer coated glass beads prepared were weighed and placed in deionised water in universal bottles in shaking water bath at 37ºC for 3 d. Changes in the dry weight of the polymer coatings were recorded. A dried sample (0.5 g) of each crosslinked polymer was stirred in an aqueous solution (20 mL) of either 2 M hydrochloric acid or 10 % (w/v) sodium hydroxide in a 50 mL conical flask at 37ºC. The polymer was examined for signs of softening and dissolution in both solutions.

RESULTS AND DISCUSSION

All polymers appeared as viscous orange colours solutions in methanol. Increasing the concentration of styrene in the polymerization mixture increased the time required for polymerization to occur (Table-1). The polymers containing higher concentrations of styrene appeared to be less readily soluble in methanol.

IR spectra of HEMA and styrene monomers contained peaks at 1640 and 1630 cm^{-1}, respectively relating to carbon-carbon double bond. But these peaks were absent in the spectra of the polymers, suggesting that polymerization of the monomers had reached completion. Co-polymer containing styrene had aromatic C–C bands at about 1500 and 1600 cm^{-1} and aromatic C–H bands at 3030 cm^{-1}, which increased in intensity with increasing concentration of styrene in the polymer. The carbonyl, 1720 cm^{-1} and hydroxyl, 3400 cm^{-1}, bands of HEMA increased in intensity with increasing percentage of HEMA in the polymers.

The distinct red colour of amaranth in nutrient broth faded gradually in cultures containing viable bacteria (Figs. 1 and 2). The colour started to disappear visibly from the lower part of each bottle forming a thin colourless layer at the bottom of each culture within 15 min of incubation. This layer gradually spread upwards until eventually the whole culture became colourless after about 2 h, in *E. faecalis* cultures and 4 h in the caecal contents. There was a lag period of *ca.* 20 min during which the reduction of amaranth was slow in the cultures of rat caecal contents. There was no change in absorbance of the control cultures (sterile nutrient broth and

sterile rat caecal contents). The concentration of *E. faecalis* was *ca.* 1.8 × 10^9 cfu/mL in the overnight cultures and 1.3 × 10^9 cfu/mL in the amaranth cultures at the end of the experiment. No growth was observed in the control cultures, which comprised either sterile nutrient broth or nutrient broth containing sterilized caecal contents.

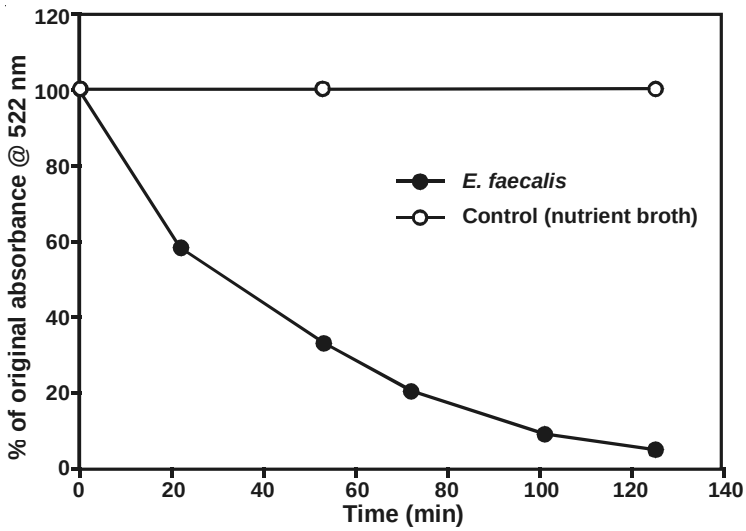

Fig. 1. Reduction of amaranth by *E. faecalis* in nutrient broth

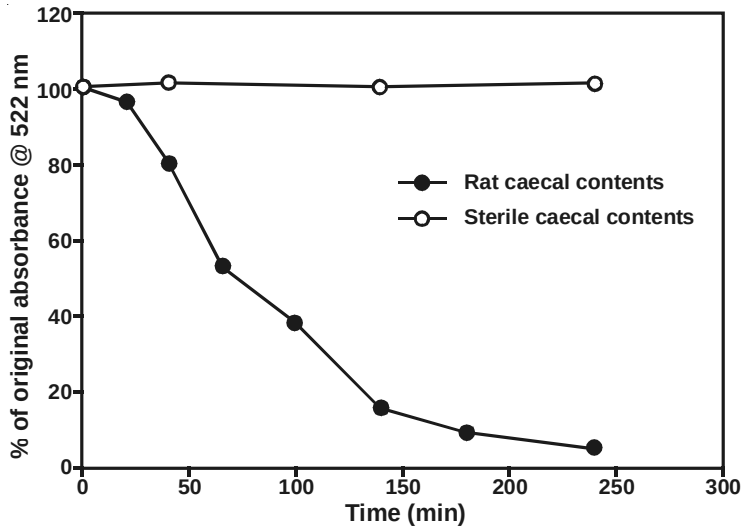

Fig. 2. Reduction of amaranth by rat caecal contents in nutrient broth

The weight of azo crosslinked polyHEMA decreased by *ca.* 20 % during incubation in the bacterial cultures. However, the changes appeared to be approximately the same in all the test and control cultures (Fig. 3). The reduction in the weight of azo crosslinked co-polymer of HEMA and styrene (ratio 95:5) was generally lower than those of crosslinked polyHEMA during incubation in the bacterial cultures. However, similar weight changes were observed in the test and control for all incubation carried out with the co-polymer (Fig. 3). Incubation of the polymers in water for 3 d with shaking resulted in separation of the polymer coating from the glass bead and dispersion in the incubation media. Crosslinked polyHEMA coating was almost completely dispersed, with only small fractions of the polymer remaining attached to the glass bead at the end of the experiment (Fig. 4). The presence of styrene in the co-polymers decreased the loss of polymer coating. In polymers containing high proportion of styrene the change in weight was very small (Fig. 4). Macroscopic examination of the polymers before and after incubation in cultures of *E. faecalis* showed some changes in the appearance of the polymer surface (Figs. 5 to 10). Polymer surfaces became irregular and less smooth during the incubation (Figs. 6, 8 and 10). There were a few microscopic cracks and holes in the polymers that contained higher concentrations of HEMA (Fig. 6), but there was no apparent disruption of gel structure in the polymer containing 80 % styrene (Fig. 10). Some debris from the bacterial culture remained attached to the polymer surface and was not removed by the washing process.

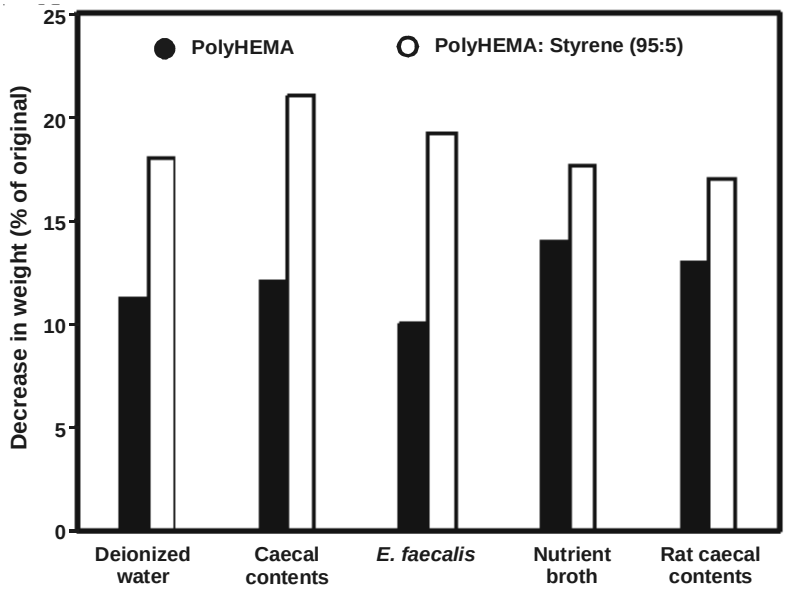

Fig. 3. Reduction of polymers in various cultures

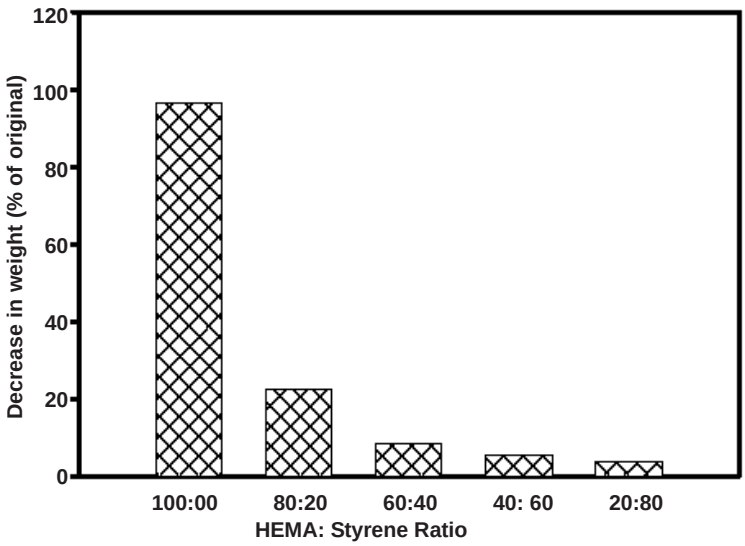

Fig. 4. Changes in the weight of polymers in distilled water after 3 d in a shaking water bath

None of the polymers dissolved in the aqueous HCl. Azo crosslinked polyHEMA dissolved completely in aqueous NaOH after *ca.* 3 min. The polymers containing styrene were less soluble and their solubility decreased with increasing concentration of styrene. Polymer with 5 and 10 % (w/v) styrene dissolve within 15 min, whilst polymer containing 40 % (w/v) or more styrene remained as solid lumps and showed no visible sign of dissolution in the aqueous NaOH media after 1.5 h.

The radical copolymerization of the vinyl monomers, HEMA and styrene, with the bifunctional crosslinker, dimethacryloyloxy benzene, is expected to form network structures. However, the actual structure of such networks is not fully understood. It is known that low molecular weight alkenes normally undergo a rapid polymerization reaction when treated with catalytic amounts of a radical initiator and the resultant polymer may have few hundred to few thousand monomer units in this chain. HEMA trends to form polymers with very high molecular weight and are readily copolymerized with many monomers because of their highly reactive double bond. Therefore, copolymerization with both styrene and the azo crosslinker was achieved readily, so that decreasing the concentration of HEMA decreased the rate of polymerization reaction (Table-1).

In this study styrene was used in the synthesis of polymers so as to control the resultant hydrophobicity. Copolymers of styrene and divinylbenzene have been reported to be hard materials that are insoluble and non-swelling in almost all solvents[6]. In the present study, the polymers containing higher concentrations of styrene and crosslinked with the divinyl

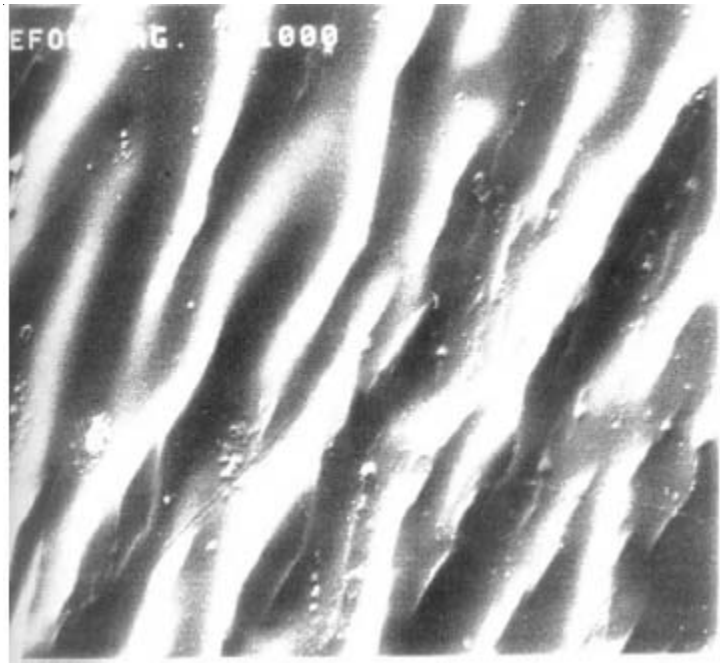

Fig. 5. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (8:2) before incubation in the culture of *E. faecalis*

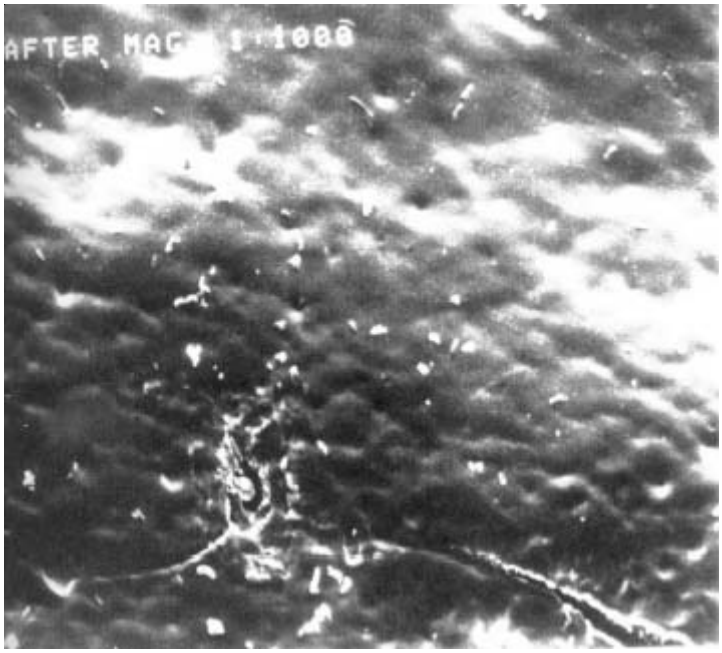

Fig. 6. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (8:2) after incubation in the culture of *E. faecalis*

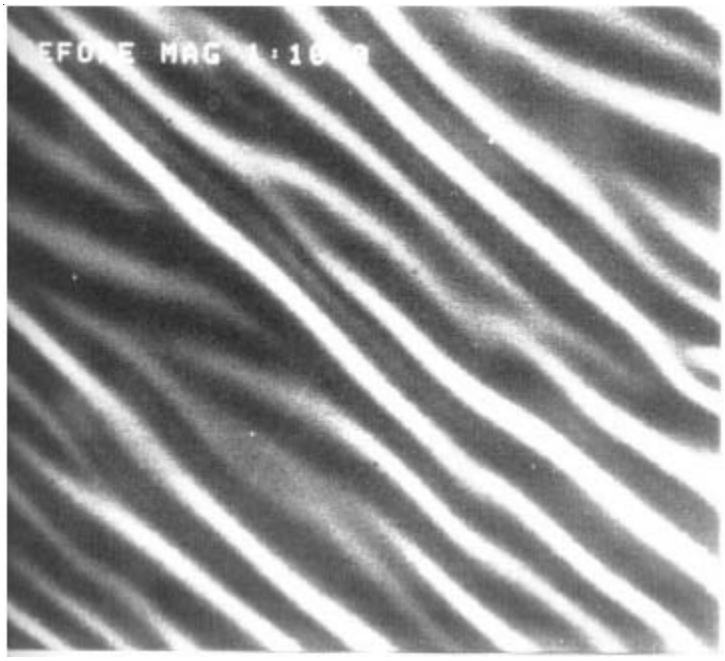

Fig. 7. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (4:6) before incubation in the culture of *E. faecalis*

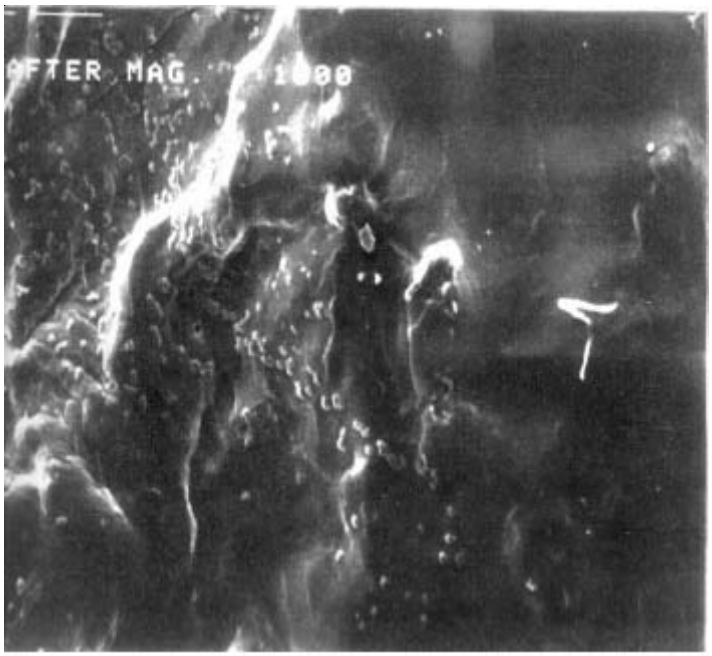

Fig. 8. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (4:6) after incubation in the culture of *E. faecalis*

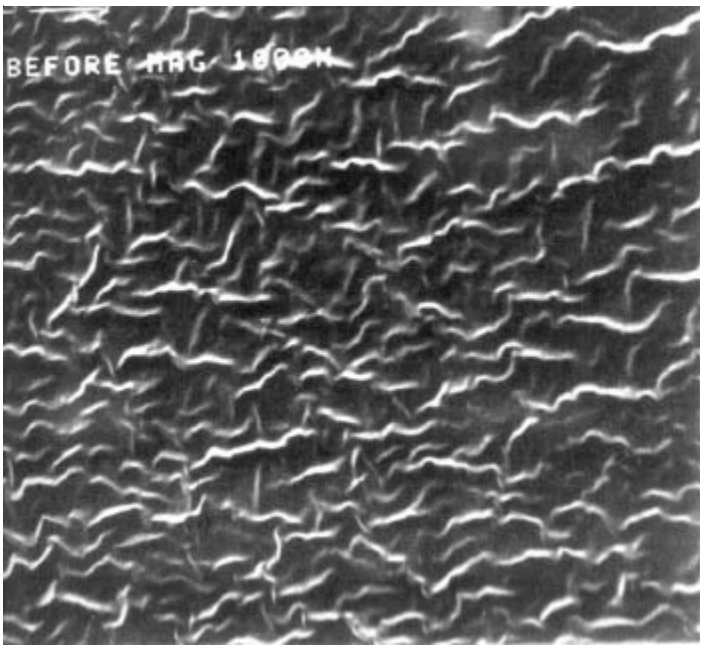

Fig. 9. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (2:8) before incubation in the culture of *E. faecalis*

Fig. 10. Macroscopic appearance of the surface of azo crosslinked polyHEMA styrene (2:8) after incubation in the culture of *E. faecalis*

azo crosslinker may be expected to have similar properties due to the similarity of their structures with that of styrene-divinylbenzene copolymers. The results obtained supported this hypothesis and the polymers with higher concentrations of styrene showed little swelling and weight loss in the aqueous media. The presence of styrene in the polymers may also provide a further opportunity to modify the properties of the polymers because polymers containing styrene are known to have reactivities similar to those of substituted benzenes and can be easily derivatised (sulphonated, nitrated, *etc.*).

From the results presented in Figs. 3 and 4, it may also be suggested that disintegration and weight loss is dependent on swellability of the polymer rather than azo reduction. It should be noted that the degree of swelling of the polyHEMA gels appeared to be insensitive to the degree of crosslinking. Secondary non-covalent bonds, probably hydrogen bonds, crosslink such polymers to form a network structure. The degree of this inter and intramolecular bonding is so high that the relatively few covalent crosslinks have little effect on the macroscopic swelling properties of the gel. Thus the secondary structure controls the behaviour of the polymer.

The molar ratio of the azo crosslinker to the other monomers in the polymer was very small, *ca.* 1:500. The amount of crosslinker that can be used in the polymer is limited by its hydrophilicity because increasing the amount of crosslinker increases the hyrdophobicity of the polymer, hindering the access of aqueous solutions to the azo bonds. On the other hand, the presence of few azo crosslinkers in the polymer will reduce the contribution of these molecules to the structure of the polymer, thereby, reducing the impact of azo reduction on polymer degradation. If the hydrophilicity of the crosslinker is increased by the addition of substituents, such as -OH, more crosslinker may be used in the polymer and, hence, azo reduction may be more effective at contributing to the degradation of the polymer.

Commercial HEMA monomer contains impurities such as ethylene glycol, methacrylic acid and ethylene dimethacrylate (DME). DME impurity in HEMA monomer cannot be removed by conventional purification methods, such as distillation. The relative contribution of each crosslinker to the polymer structure was not investigated, however, the DME crosslinkers are likely to have a greater role in holding the polymer chains together because the concentration of DME impurity was probably higher than the concentration of azo crosslinker. Consequently, breakdown of DME may be expected to have greater influence than azo reduction as a measure of azo reduction in azo polymers, because it may be argued that even if the azo bond are cleaved, the DME crosslinks can still hold the polymer together. However the weight reduction reported in the present study are unlikely to have been caused or aided significantly by chemical or

enzymatic cleavage of the crosslinks since polymer weight changes in bacterial cultures were not higher than those observed in deionised water. A more plausible explanation for the reduction in the weight of the polymers is the possible loss, by leaching out into aqueous media, of unpolymerised monomers and low molecular weight polymer chains from the polymer. Further loss of polymer could occur after the hydration and expansion of the polymer network due to dislodgment of the polymer fragments from the stretched polymer. Such break up of the polymer structure assisted by physical abrasion in the shaken water bath was probably responsible for the nearly complete loss of crosslinked polyHEMA. Similar polymer disruptive factors, *i.e.* aqueous media and mechanical abrasion, are likely to be encountered by an azopolymer coated solid dosage from during transit in the GIT and may lead to the break up of the polymer coating.

Macroscopic examination of the polymers showed some changes in the appearance of the polymer surface during incubation in cultures of *E. faecalis*. The polymer surface became irregular and less smooth during the incubation (Figs. 5 to 10). However, there was very little difference in weight reduction between these polymers and those incubated in control cultures (Fig. 3). It is known that the swelling of polymers containing HEMA markedly alter the morphology of the polymer surface, which is in contact with an aqueous biological system. Therefore, the changes in the surface morphology may not be good indication of polymer degradation by azo reduction. Furthermore, microscopic and macroscopic changes in the appearance of the polymers can also be caused by breakdown of the polymer backbone and cannot be taken as proof of azo reduction in the polymers.

Van den Mooter *et al.*[12] presented spectrophotometric data, showing the reduction of absorbance of an azo crosslinked polymer at the λ_{max} of the crosslinker during anaerobic in the culture of human faeces. The absorbance decreased by 60 % of the original value over 5 d. Although it is concluded in present studies that azo reduction took place in the polymers and that it could be used to trigger drug release from azo polymer drug delivery devices in the colon. The rate of reduction was probably too slow to result in sufficient polymer degradation within the normal residence time of the drug in the proximal colon. Furthermore, the results of the current study showed that polymer weight loss could occur on incubation in aqueous media, such a reduction in the amount of polymer can explain the reduction in absorbance when the polymer was redissolved in a solvent after incubation.

Azo bond cleavage in bacterial cultures is a reductive process and being inhibited by the presence of oxygen. Therefore, using an anaerobic culture medium would be expected to enhance azo reduction and also momic the anaerobic environment of the colon. However, most anaerobic culture

media contain chemical reducing agents, such as sodium thioglycollate broth, which are included in the media in order to reduce dissolved oxygen and create an anaerobic environment. Amaranth was shown to be reduced in thioglycollate broth and this chemical reaction was speeded up by the heat of sterilization. Therefore, nutrient broth was chosen as the growth medium because it did not have any effect on the colour of amaranth and it formed a nearly clear solution which caused little interference with the absorbance of amaranth at 522 nm.

Attempts were made to exclude as much oxygen as possible from the cultures. The solutions and media were boiled before use. The container were filled to the top with the medium to reduce the amount of trapped air and effective seals were used to reduce air flow into the bottles. Oxygen pressure was reduced to a value low enough to reduce amaranth effectively (Figs. 1 and 2). Reduction was fastest at the bottom of the bottles where the oxygen pressure is likely to be lowest.

Furthermore, since *E. faecalis* is facultative anaerobe it can be more conveniently handled and manipulated than strict anaerobic organism, therefore, it was chosen for this study. No numerical value for the concentration of *E. faecalis* in the colon has been cited, but the total concentration of enterococci is in the region of 10^7 cell/g in man and 10^6 cell/g in the rat[21]. Under the conditions employed in this study amaranth was almost entirely reduced within 2 h (Fig. 1).

The ability to reduce polymeric azo compounds is not limited to a particular component of the intestinal microflora, but rather seems to be shared by all the bacteria to a varying degree. The use of caecal contents rather than pure bacterial cultures may be advantageous in the study of polymer degradation because the caecal contents, in addition to a wide range of colonic bacteria, also contain the nonbacteria luminal contents, including extracellular enzymes, electron carriers and solid material which may contribute to the polymer erosion by physical abrasion in the colon. In order to simulate more closely the *in vivo* situation, the experiments were repeated using cultures of rat caecal contents. The results showed that there was no difference in weight loss between polymer suspended in caecal contents, *E. faecalis* cultures, or the controls such as sterilized caecal contents, nutrient broth and deionised water (Fig. 3), although amaranth was reduced by the viable caecal contents and *E. faecalis* but not by the control cultured (Figs. 1 and 2). This, further, suggested that the polymer degradation was not mediated by the bacteria.

As the colonic bacteria are incubated for prolonged periods the relative proportions of each organism will change. Furthermore, some of the strict anaerobic bacteria of the caecal contents may be killed by the short exposure to air upon their removal from the animal. Therefore,

standardization of enzyme activity of caecal bacteria contents is far more complex than that of a culture containing only one type of bacteria.

The reduction of amaranth appeared to be slower in caecal contents than in the cultures of *E. faecalis* (Figs. 1 and 2). This may be due to the death of the strict anaerobes, which form a significant proportion of the caecal microflora. Also, the substantial change of environment when the bacteria were moved from the caecum to the nutrient broth can adversely affect the growth and metabolic activity of the bacteria. Fig. 2 shows a "lag" period of slow reduction of amaranth before a more rapid reduction commenced. This was probably time taken by the colonic bacteria to adapt to the new medium and resume growth.

The bacteria cultures reduced the azo bond in amaranth but apparently did not reduce the insoluble azo crosslinker in the polymer. Steric effects of the polymer may decrease the accessibility of the reducing agents to the azo bond so that readily reduced than the azo bond in amaranth.

Although *in vivo* reports of drug release from azopolymer coated oral dosage forms in the lower gastrointestinal tract appear to be consistent with the degradation of the azo crosslinked polyHEMA[22], the results of the current study suggest that the mechanism of release was unlikely to involve microbial azoreduction. A more likely explanation is that hydrolysis of the HEMA ester groups in the azo polymer backbone occurred and polymer degradation was further aided by swelling in the aqueous media of the GIT and mechanical erosion during transit in the GIT.

Conclusions

The experiments described in this study were originally intended to examine the importance of polymer hydrophobicity, prior to further studies aimed at appropriate polymer selection and formulation. However, the results indicated that the azopolymers containing 2,2'-dimethacryloxyazo benzene as crosslinker were not reduced by the azoreductase activity of the bacterial systems shown to reduce amaranth. The azo crosslinkers have a central role in the drug release trigger mechanism from the proposed delivery system. The detailed study of azo compounds may reveal information about the relationship between the structure and the ease of reduction of these compounds.

Copolymerization of HEMA and styrene with the bifunctional crosslinker forms network structures. The role of styrene was to control the hydrophobicity of polymers so that the polymers with higher concentrations of styrene showed little swelling and weight loss in the aqueous media. Disintegration and weight loss is dependent on swellability of the polymer rather than azo reduction. Degree of swelling of the polyHEMA gels appeared to be insensitive to the degree of crosslinking. The molar ratio of the azo crosslinker to the other monomers in the polymer was very

small to reduce the contribution of these molecules to the structure of the polymer, thereby, reducing the impact of azo reduction on polymer degradation. The polymer surface became irregular and less smooth during the incubation. Furthermore, microscopic and macroscopic changes in the appearance of the polymers can also be caused by breakdown of the polymer backbone and cannot be taken as proof of azo reduction in the polymers. Azo bond cleavage in bacterial cultures is a reductive process and being inhibited by the presence of oxygen. This, further, suggested that the polymer degradation was not mediated by the bacteria. The results of the current study suggest that the mechanism of release was unlikely to involve microbial azo reduction.

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