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# Synthesis and bacterial degradation of an azopolymer

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**Abstract**

Azopolymers were synthesised with differing degrees of hydrophobicity, from 2-hydroxyethylmethacrylate (HEMA), styrene and 2,2′-dimethylacryloyloxyazobenzene as azo crosslinker. Bacterial degradation of the series of polymers was assessed using a pure culture of the colonic organism *Enterococcus faecalis* and rat caecal contents. Polymer degradation was determined in terms of weight loss on polymer coated glass beads and using scanning electron microscopy after incubation. Similar weight loss occurred on incubation of polymers in both bacterial cultures and non-bacterial control. The presence of styrene was found to decrease the amount of weight loss. The polymer surfaces showed microscopic cracks and holes after incubation, again, this phenomenon was less pronounced with increasing styrene content. As there was no increase in polymer degradation in the presence of azo reducing microorganisms, the results of this study suggest that these polymers are degraded by mechanisms other than azo reduction. 

*Keywords:* Azopolymers; HEMA; Styrene; 2,2′-Dimethylacryloyloxyazobenzene; Bacterial degradation

## 1. Introduction

Hydrophilic polymer networks (hydrogels) derived from polymers or copolymers of methacrylic esters, such as 2-hydroxyethylmethacrylate (HEMA), have been shown to be well tolerated by biological systems and have many biomedical applications (Kim, 1983; Park and Park, 1996; Wheeler et al., 1996). Crosslinked polymers of HEMA are insoluble in water but, due to the presence of an alcohol group in the HEMA monomer, they have some limited compatibility with water and swell in aqueous media to allow the diffusion of aqueous solutions within the polymer (Macret and Hild, 1982).

Saffran et al. (1986) suggested that polymers containing HEMA, as a co-monomer, and azoaromatic crosslinkers could be used for colon-specific drug delivery as the azoaromatic crosslinkers would be degraded by colonic bacteria. These early studies stimulated intense research interest in the field of azopolymers (Shantha et al.,

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1995; Yeh et al., 1995; Schacht et al., 1996; Bragger et al., 1997; David et al., 1997; Van den Mooter et al., 1997; Kakoulides et al., 1998). However, the early studies undertaken by Saffran et al. (1986) were poorly controlled, showed variable in vivo results, and the method employed to synthesise the azo crosslinker has been impossible to repeat by workers in several laboratories. The variable in vivo results were explained to some extent in a later publication (Saffran, 1992) when it was disclosed that the integrity of the azopolymer capsules used for in vivo work had been found to be unreliable.

Pradny and Kopecek (1990) 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. Van den Mooter et al. (1992) synthesised and studied a series of azopolymers consisting of HEMA and methylmethacrylate (MMA) and suggested that the variable in vivo results of Saffran et al. (1986) may have been due to insufficient hydrophilicity of the polymers.

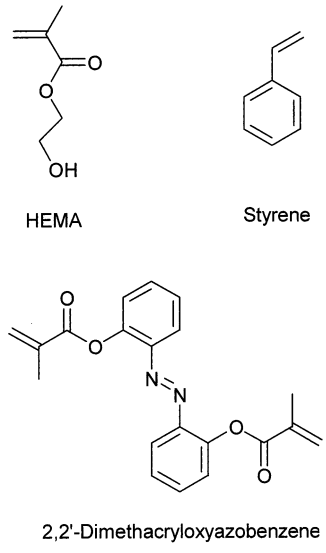


Fig. 1. Chemical structures of monomers and crosslinker.

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. While there is extensive information available on biodegradation of polymers by hydrolysis, relatively little is known about biodegradation of azopolymers. Although 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 (Walker, 1970; Brown, 1981), there is no reliable evidence to suggest that the insoluble azopolymers of the type proposed by Saffran et al. (1986) are degradable through azo reduction by biological systems.

In the initial studies on polymers of HEMA and styrene (Saffran et al., 1986), the in vitro results may be criticised on the basis of the controls which were used. Colorimetric analysis of degradation may be complicated by the increased opacity of the gel as it swells; no control without microflora was reported. Similarly, using electron microscopy, the incubated azopolymer was compared to non-incubated polymer rather than to the polymer incubated in medium without bacteria. Also, elongation of the polymer may have been due to disruption of chemical bonds within the polymer chain rather than reduction of the azo crosslinks; controls of polymer in which the azo crosslinker is replaced by a similar non-reducible crosslinker would be necessary to prove that changes in physical characteristics are due specifically to the reduction of azo bonds.

The purpose of this study was to investigate, using appropriate controls, 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 dimethacryloyloxy-azobenzene as azo crosslinker (see Fig. 1 for structures) using the procedure described by Saffran et al. (1986).

Table 1
Polymerisation times of azo crosslinked co-polymers

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

## 2. Materials and methods

### 2.1. General materials

Benzoyl peroxide, 2-hydroxyethylmethacrylate (HEMA) and styrene were obtained from Aldrich (Gillingham, Dorset, UK). HEMA and styrene contained polymerisation inhibitors, hydroquinone monomethyl ether (300 ppm) and *p*-tert-butylcatechol (10 ppm), respectively. 2,2′-Dimethacryloxyazobenzene was kindly donated by Ciba (Horsham, W. Sussex, UK). Amaranth was obtained from BDH (Poole, Dorset, UK).

### 2.2. Bacteria

*Enterococcus faecalis* NCIMB775 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK and was maintained on nutrient agar at 4°C and subcultured at ~3-monthly intervals. Caecal bacterial content was obtained from male Wistar outbred rats, fed on SDS Expanded Diet, No. 1.

### 2.3. Microbiological media

Nutrient broth, Oxoid CM1, thioglycollate broth, and phosphate buffered saline (PBS) tablets were obtained from Unipath (Basingstoke, Hampshire, UK). They were prepared and sterilised according to the manufacturer's instructions.

Nutrient agar DM179 was obtained from Mast Laboratories (Merseyside, UK), and was prepared and sterilised according to the manufacturer's instructions.

Universal culture bottles of total capacity of 26.5 ml, with rubber lined aluminium screw-caps were used to contain bacterial cultures. Glass beads, 1 cm in diameter with five small projections (3 mm in length) and a glass handle, were manufactured at the University of Brighton.

IR spectra were recorded on Infrared Spectrophotometer, 157G, Perkin-Elmer (Buckinghamshire, UK). UV/VIS spectra were recorded on a Lambda II Spectrophotometer, Perkin-Elmer (Buckinghamshire, UK).

### 2.4. Polymer synthesis

Before polymerisation HEMA was distilled under reduced pressure (67°C, 3.5 mmHg) to remove the polymerisation inhibitor, *p*-tert-butylcatechol. The inhibitor in styrene was also removed by extraction into an equal volume of 5% w/v aqueous NaOH.

The approach used by Saffran et al. (1986) for polymer synthesis was adopted. In brief, methanol, 10 ml, 2,2′-dimethacryloxyazobenzene, 0.050 g, benzoyl peroxide, 0.120 g and the monomers, 10 g in total (HEMA, 2–10 g, and styrene, 0–8 g), were placed in a round bottom flask and heated at 80°C, with stirring, until polymerisation was complete. The polymers were precipitated by addition of an excess of water, purified by repetitive precipitation from methanol, washed with water and ethanol and finally dissolved in 5 ml of methanol and transferred to a 100-ml conical flask.

A small amount of the methanolic solution of each polymer was poured on a siliconised circular glass slide to form a thin film of ~2 cm diameter and was allowed to dry at 40°C. Infrared spectra of the dried films were obtained.

All polymers appeared as viscous orange coloured solutions in methanol. Increasing the concentration of styrene in the polymerisation mixture increased the time required for polymerisation 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 bonds, but these peaks were absent in the spectra of the polymers. Co-polymers containing styrene had aromatic C-C bands at $\sim$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 hydroxy, 3400 $cm^{-1}$, bands of HEMA increased in intensity with increasing percentage of HEMA in the polymers.

### 2.5. Preparation of overnight 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 37°C overnight (for not less than 15 h). This dense bacterial suspension was used to inoculate other cultures.

Viable counts of bacterial cultures were measured by performing serial dilutions of the culture in sterile 0.01 M phosphate buffered saline (PBS) at pH 7.4, and using the standard surface spread technique.

### 2.6. Reduction of amaranth in cultures of E. faecalis

A total of 36 universal bottles each containing a solution of amaranth (0.01% w/v) in nutrient broth CM1 (25 ml) were sterilised in an autoclave at 121°C for 15 min. The solutions were maintained at 37°C, inoculated with 0.4 ml of an overnight culture of *E. faecalis* and incubated at 37°C. Then six bottles were taken out of the incubator at each time interval and a sample (5 ml) from each bottle was centrifuged at $3000 \times g$ for 10 min at 4°C 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.

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.

### 2.7. 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 37°C. In control experiments the caecal contents were wrapped in aluminium foil and sterilised in an autoclave at 121°C for 15 min before being added to the solution of amaranth in nutrient broth. Sample collection and absorbance measurements were carried out as described above.

### 2.8. Incubation of polymers in bacterial cultures

Uniform-size glass beads, 1 cm in diameter, were weighed and sterilised by soaking in 70% v/v ethanol for 30 min. 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 maximise 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 sterilised nutrient broth, 23 ml, in a universal bottle, inoculated with 0.4 ml of an overnight culture of *E. faecalis* and incubated at 37°C for 10 days. The bead was then removed from the culture, washed under a gentle stream of distilled water, dried at 60°C 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) sterilised rat caecal contents in nutrient broth; and (c) deionised water.

### *2.9. Effect of shaking aqueous media on the polymers*

Polymer coated glass beads, were weighed and placed in deionised water in universal bottles in a shaking water bath at 37°C for 3 days. Changes in the dry weight of the polymer coatings were recorded as described above.

### *2.10. Effect of aqueous acid and alkaline media on the azo polymers*

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 37°C. The polymer was examined for signs of softening and dissolution in both solutions.

## 3. Results

### *3.1. Reduction of amaranth in the bacterial cultures*

The distinct red colour of amaranth in nutrient broth faded gradually in cultures containing viable bacteria (Figs. 2 and 3). 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 $\sim 2$ h, in *E. faecalis* cultures (Fig. 2), and 4 h in the caecal contents (Fig. 3). There was a lag period of $\sim 20$ 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 $\sim 1.8 \times 10^9$ CFU $ml^{-1}$ in the overnight cultures and $1.3 \times 10^9$ CFU $ml^{-1}$ in the amaranth containing 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 sterilised caecal contents.

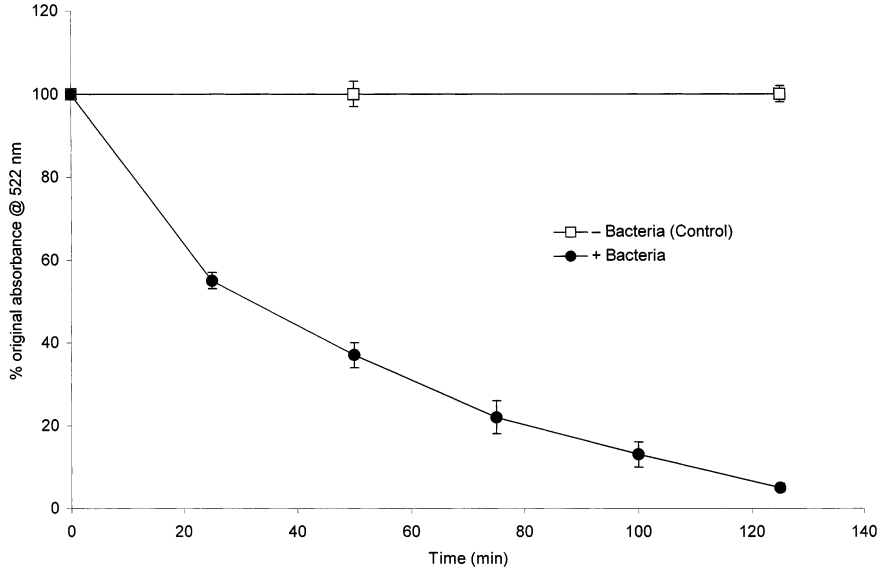


Fig. 2. Reduction of amaranth by *E. faecalis*. Mean ± S.D. ($n = 6$).

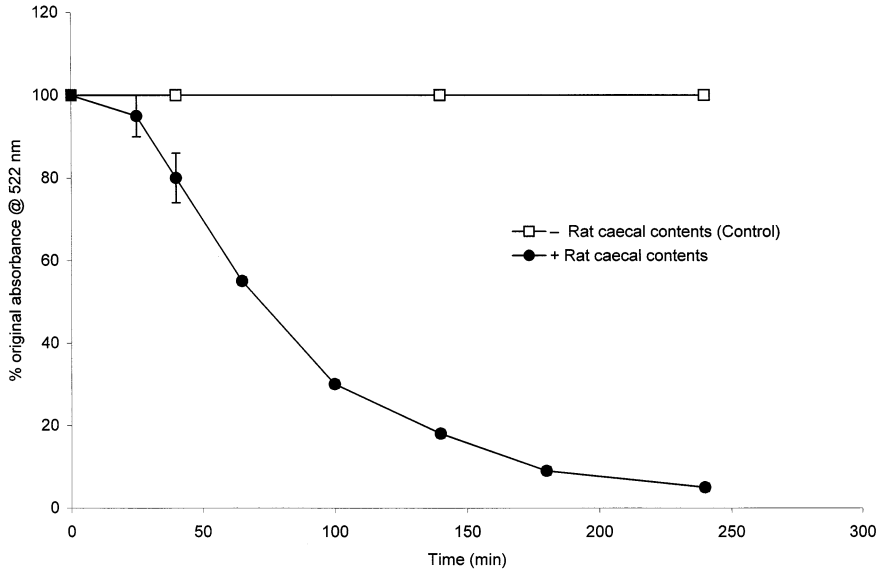


Fig. 3. Reduction of amaranth by rat caecal contents. Mean ± S.D. ($n = 6$).

### 3.2. Effect of bacterial cultures on the polymers

The weight of azo crosslinked polyHEMA decreased by ~20% during incubation in the bacterial cultures. However, the changes appeared to be approximately the same in all the test and control cultures (Fig. 4).

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 cultures for all incubations carried out with the co-polymer (Fig. 4).

Incubation of the polymers in water for 3 days with shaking resulted in separation of the polymer coating from the glass bead and dispersion in the

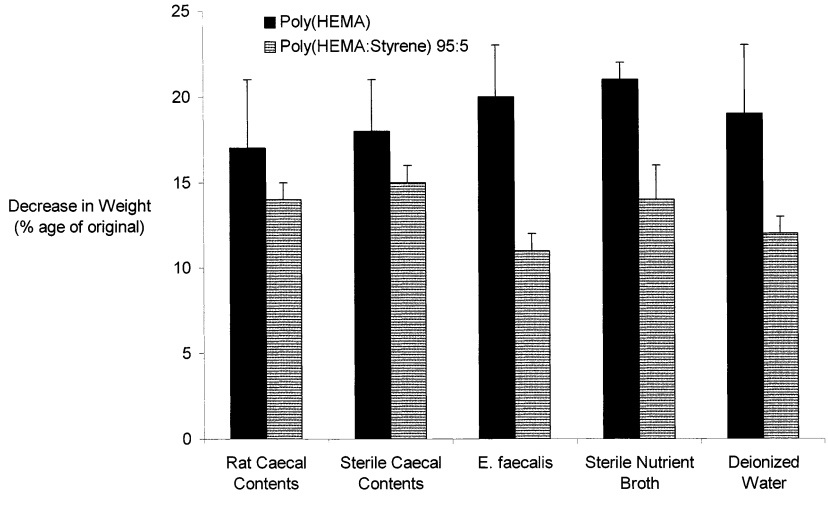


Fig. 4. Degradation of azo crosslinked polymers in different media. Mean ± S.D. ($n = 6$).

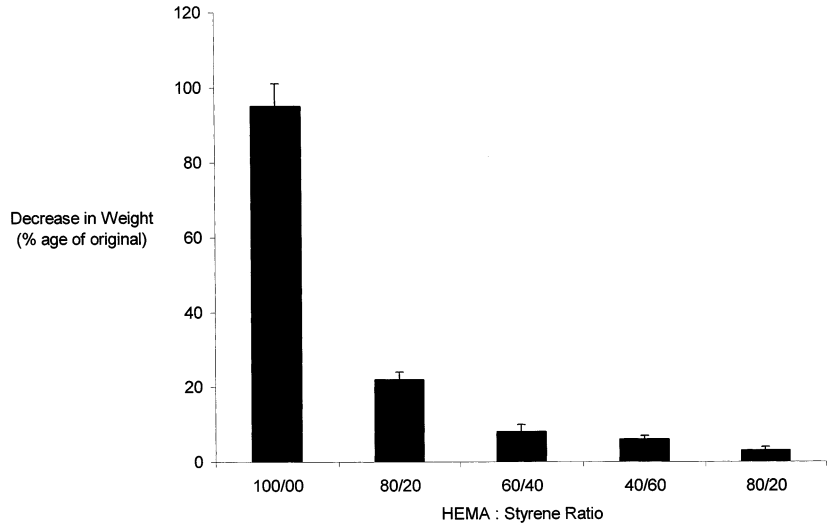


Fig. 5. Dissolution of different azo crosslinked polymers after 3 days in water. Mean ± S.D. ($n = 6$).

incubating 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. 5). 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. 5).

Microscopic examination of the polymers before and after incubation in cultures of *E. faecalis* showed some change in the appearance of the polymer surface (Fig. 6a–f). Polymer surfaces became irregular and less smooth during the incubation (Fig. 6b, d, f). There were a few microscopic cracks and holes in the polymers that contained higher concentrations of HEMA (Fig. 6b), but there was no apparent disruption of gel structure in the polymer containing 80% styrene (Fig. 6f). Some debris from the bacterial culture remained attached to the polymer surface and was not removed by the washing process.

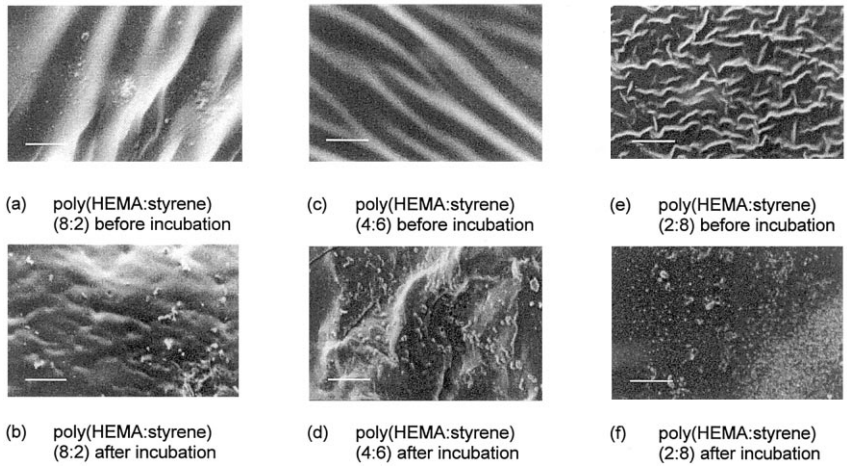


Fig. 6. Scanning electron micrographs of polymers before and after incubation in cultures of *E. faecalis* (bar indicates 10 μm).

### 3.3. Effect of aqueous acid and alkaline media on the azo polymers

None of the polymers dissolved in the aqueous HCl. Azo crosslinked polyHEMA dissolved completely in aqueous NaOH after ~3 min. The polymers containing styrene were less soluble and their solubility decreased with increasing concentration of styrene. Polymers with 5 and 10% w/v styrene dissolved within 15 min, whilst polymers containing 40% w/v or more styrene remained as solid lumps and showed no visible sign of dissolution in the aqueous NaOH media after 90 min.

## 4. Discussion

The radical copolymerisation of the vinyl monomers, HEMA and styrene, with the bifunctional crosslinker, dimethacryloyloxyazobenzene, in this study is expected to form network structures (Hild and Rempp, 1981). In common with most previous studies on azo polymers (Saffran et al., 1986; Cheng et al., 1994), the molecular size of the polymers in this study was not determined. However, it is known that low-molecular-weight alkenes normally undergo a rapid polymerisation reaction when treated with catalytic amounts of a radical initiator and the resultant polymer may have anything from a few hundred to a few thousand monomer units in the chain. Methacrylates, such as HEMA, tend to form polymers with very high molecular weights and are readily copolymerised with many monomers because of their miscibility with both oil-soluble and water-soluble monomers (Kumar, 1987). Therefore, copolymerisation with both styrene and the azo crosslinker was achieved readily. Decreasing the concentration of HEMA decreased rate of polymerisation 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 (Munk, 1989). In the present study the polymers containing higher concentrations of styrene and crosslinked with the divinyl 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.) (Munk, 1989).

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. This is in agreement with the findings of Van den Mooter et al. (1993) who suggested that swellability of the polymer, which was dependent on the balance between the hydrophobic and hydrophilic monomers, was a more important factor in its degradation than the nature of the azo crosslinks. The authors also reported that the 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, ~1:500. The amount of crosslinker that can be used in the polymer is limited by its hydrophobicity because increasing the amount of crosslinker increases the hydrophobicity of the polymer, hindering the access of aqueous solutions to the azo bonds. On the other hand, the presence of too 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.

Other molar ratios of 'crosslinker:other monomers' have been used, e.g. 1:250 (Van den Mooter et al., 1993) and 1:200 (Saffran et al., 1986), but capsule coatings made of such polymers showed imperfections and allowed perme-

ation of water when they were immersed in an aqueous media (Saffran, 1992). Crosslinking of polymers tends to increase structural rigidity, because the individual chains can no longer slip over each other but are instead locked together into immense single molecules. The discontinuity in the coatings was probably caused by the reduced flexibility of the polymer due to the high degree of crosslinking. Such cracks are likely to increase in size and number under the physical pressure on the dosage form during its passage through the GIT.

Commercial HEMA monomer contains impurities such as ethylene glycol, methacrylic acid and ethylene dimethacrylate (DME). Although the suppliers of HEMA used in this investigation were unable to provide accurate values for the concentration of the impurities, figures as high as 4% w/w for bifunctional monomers in commercial HEMA have been reported (Macret and Hild, 1982). Ethylene dimethacrylate, a by-product of HEMA synthesis, is a bifunctional monomer which can act as a crosslinker when HEMA is polymerised to form a network structure, whereas pure HEMA has been shown to form linear polymers (Macret and Hild, 1982). Crosslinked polyHEMA has, in fact, been synthesised by deliberate addition of various amounts of DME to HEMA, for biomedical applications, such as contact lenses (Levowitz et al., 1968; Ilovsky et al., 1972).

DME impurity in HEMA monomer cannot be removed by conventional purification methods, such as distillation, although a chromatographic procedure has been described for the separation of DME and HEMA (Macret and Hild, 1982). Azo crosslinked polymers of HEMA as described here and by Saffran et al. (1986), therefore, also contain crosslinks of DME and the complete breakdown of the polymer network would be dependent on the cleavage of both types of crosslinks. While the azo crosslinkers may be split by the reduction of their azo bond to corresponding amines, DME crosslinkers are cleaved by chemical or enzymatic hydrolysis.

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 on polymer degradation. This may raise concerns over the use of weight reduction as a measure of azo reduction in azo polymers, because it may be argued that even if the azo bonds are cleaved, the DME crosslinks can still hold the polymer together. However the weight reduction reported in the present study is 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 the aqueous media, of unreacted monomers and low molecular weight polymer chains from the polymer which remained entrapped during the precipitation of the polymer; more stable coatings may possibly be obtained by better purification of the product to remove these low molecular weight entities. Such break-up of the polymer structure assisted by physical abrasion in the shaken water bath was probably responsible for the near complete loss of crosslinked polyHEMA (Fig. 5). Similar polymer disruptive factors, i.e. aqueous media and mechanical abrasion, are likely to be encountered by an azopolymer coated solid dosage form during transit in the GIT and may lead to the break-up of the polymer coating. However, the important observation of this work is that no increase in the rate of polymer degradation was observed in the microbial cultures which had been shown to have extensive azo reducing capacity.

Microscopic 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 (Fig. 6a–f). However, there was very little difference in weight reduction between these polymers and those incubated in control cultures (Fig. 4). Similar changes in the appearance of azo polymers have also been reported by other investigators

and have been attributed to polymer degradation due to cleavage of azo bonds of the polymers by the bacteria (Saffran et al., 1986; Van den Mooter et al., 1992). However, it is known that the swelling of polymers containing HEMA markedly alters the morphology of the polymer surface, which is in contact with an aqueous biological system (Kim, 1983). Therefore, the changes in the surface morphology may not be a 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.

Some workers have investigated the swellability or the viscosity of the polymers before and after in vitro or in vivo incubation with caecal contents (Van den Mooter et al., 1993; Cheng et al., 1994). These experiments are unable to distinguish the changes in macromolecular structure which occur to the polymer as a result of azo reduction from that which would occur upon degradation of the polymer by some other mechanism, such as the hydrolysis of hydroxyethyl esters. Although these earlier studies provide useful information relating to the caecal degradation of such polymers it has not been conclusively demonstrated that degradation occurs as a consequence of azo reduction.

Spectrophotometric methods have been used to monitor azo reduction in the polymers, which often exhibit the colour of the azo crosslinker (Saffran et al., 1990; Van den Mooter et al., 1995). However, these hydrogels have a tendency to become opaque as the gel swells as a consequence of changes in refractive index of the gel; such a change in opacity may mask any colour change attributable to simultaneous reduction of the azo bonds within a hydrogel. Colorimetric methods are also unable to distinguish between reduction of an azo bond to the intermediate hydrazo moiety, a process which is often reversible, from the complete reduction of this species to the free amines. Permeability studies on thin films of azo polymers have been used to show that permeability of the films to small molecules increases on incubation in bacterial cultures (Van den Mooter et al., 1992). However, the results do not prove that microbial azo reduction was responsible for the increased permeability.

Azo bond cleavage in bacterial cultures is a reductive process and has been reported to be inhibited by the presence of oxygen (Walker and Ryan, 1971; Azad Khan et al., 1983). Therefore, using an anaerobic culture medium would be expected to enhance azo reduction, and also mimic the anaerobic environment of the colon. However, most anaerobic culture media contain chemical reducing agents, such as sodium thioglycollate in 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 accelerated by the heat of sterilisation. 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; containers 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 (Fig. 2). Reduction was fastest at the bottom of the bottles where the oxygen pressure is likely to be lowest.

A model was required to investigate the degradation of the polymers. *E. faecalis*, which is commonly found in the large intestine, is documented as one of the most effective organisms for reducing azo dyes (Walker and Ryan, 1971; Shantha et al., 1995). Furthermore, since *E. faecalis* is a facultative anaerobe it can be more conveniently handled and manipulated than strict anaerobic organisms, therefore, it was chosen for this study. Under the conditions employed in this study amaranth was almost entirely reduced within 2 h (Fig. 2).

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 bacteria to a varying degree (Brown,

1981). 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 non-bacterial luminal contents, including extracellular enzymes, electron carriers and solid material which may contribute to the polymer erosion by physical abrasion in the colon. The results showed that there was no difference in weight loss between polymer suspended in caecal contents, *E. faecalis* cultures, or the controls (sterilised caecal contents, nutrient broth and deionised water) (Fig. 4), although amaranth was reduced by the viable caecal contents and *E. faecalis* but not by the control cultures (Figs. 2 and 3). This further suggested that the polymer degradation was not mediated by the bacteria.

The reduction of amaranth appeared to be slower in caecal contents (Fig. 3) than in the cultures of *E. faecalis* (Fig. 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.

The bacterial 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 it is less 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 poly-HEMA (Hastewell et al., 1991), the results of the current study suggest that the mechanism of release was unlikely to involve microbial azo reduction. A more likely explanation is that some hydrolysis of the HEMA ester groups in the azo polymer backbone occurs and polymer degradation is further aided by the consequential increased swelling in the aqueous media of the GIT and mechanical erosion during transit in the GIT.

## 5. Conclusion

The results of this study indicated that azopolymers containing 2,2′-dimethacryloxyazobenzene as crosslinker are not reduced by the azoreductase activity of the bacterial systems shown to reduce amaranth. This finding appears to contradict the reports of reduction by intestinal bacteria of azo bonds in polymers crosslinked with a similar crosslinker, 4,4′-divinylazobenzene, (azostyrene), both in vivo and in vitro (Saffran et al., 1986). A more detailed study of the earlier reports in the literature indicates that little consideration was given by the previous workers to either the ease or extent of reduction of the chosen azo crosslinkers. As no control experiments, using a similar polymer coating without the azo crosslinkers, were reported in any of the previous studies, the possibility of degradation and chemical breakdown of the HEMA polymer backbone remains uninvestigated.

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