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Synthesis and in vitro biodegradation of poly(ether-ester) azo polymers designed for colon targeting

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Abstract

A number of poly(ether-ester) azo polymers consisting of various concentrations of 4-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}benzoic acid (HEEEBA), 4-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}phenylazobenzoic acid (HEEEPABA) and either 12-hydroxydodecanoic or 16-hydroxyhexadecanoic acid were synthesized. The polymers were assessed in order to determine whether they are biodegradable by the azoreductase present in the rat caecum. The polymers containing HEEEBA showed satisfactory degradation (up to 48%), while those without showed poor degradation, due to poor wettability. When 1% polysorbate 80 (a wetting agent) was added to the polymers in the degradation medium, all polymers showed excellent degradation. Azo polymers with HEEEBA have the potential to be used as colon-specific drug release materials.

Keywords: Azo polymer; Colon targeting; Selective drug delivery; Biodegradable polymer; Azoreductase; Polycondensation

1. Introduction

With the advances of new biologically active peptide and protein drugs under development in the biotechnology field, there is a growing interest in utilizing the colon as a site for drug absorption. Indeed, the colon may be the best site for peptide delivery because of the long residence time and the low digestive, enzymatic activities. Another application for colon targeting may be found in the topical treatment of disorders of the colon such as Crohn's disease and colitis.

Although colon-specific drug delivery has attracted much interest during the past decade, today there is still no satisfactory system available to deliver drugs to the large intestine. Several potential colonic targeting systems, based on pH-sensitive polymers (Lehmann, 1975), timed-release preparations such as the Pulsincap® system (Rashid, 1990), biodegradable matrix systems and hydrogels (Brondsted and Kopecek, 1991; Kopecek et al., 1992; Rubinstein et al., 1992, 1993), biodegradable coatings (Saffran et al., 1986; Van den Mooter et al., 1993), or prodrugs (Friend and Chang, 1985), some of which have bioadhe-

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sive properties (Kopeckova et al., 1994), have been reported in the literature.

In this paper we present the synthesis of new poly(ether-ester) azo polymers designed for colonic targeting. The polymers are based on 4-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}benzoic acid (HEEEBA), 4-{2-[2-(2-hydroxyethoxy)-ethoxy]ethoxy}phenylazobenzoic acid (HEEEP-ABA), and 16-hydroxyhexadecanoic acid or 12-hydroxydodecanoic acid, and were synthesized by polycondensation.

The biodegradation of the polymers was studied by following the decrease in concentration of azo bonds as a function of anaerobic incubation time in a bacterial cell-free extract possessing azoreductase activity.

2. Materials and methods

2.1. Chemicals

4-Aminobenzoic acid and 4-hydroxybenzoic acid (both from Janssen Chimica, Geel, Belgium), phenol, sodium nitrite (Baker Chemicals, Deventer, The Netherlands), 2-[2-(2-chloroethoxy)-ethoxy]ethanol, 16-hydroxyhexadecanoic acid, and 12-hydroxydodecanoic acid (all Aldrich-Chemie, Steinheim, Germany) were used without any further purification.

Nicotinamide adenine dinucleotide phosphate, benzyl viologen, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase, all used to prepare the cell-free extract, were purchased from Sigma (Bornem, Belgium).

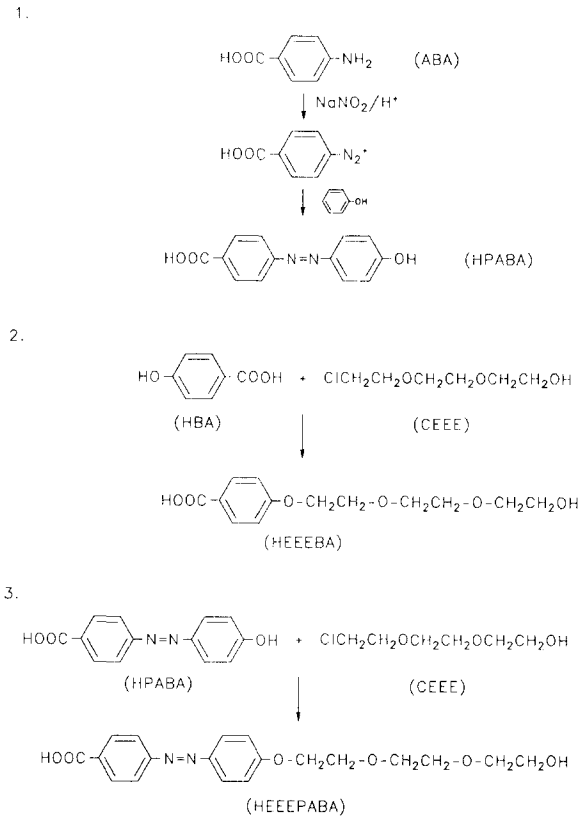
2.2. Instrumentation

UV spectra were recorded using an HP84752A diode array spectrophotometer equipped with an HP vectra 286/12 computer (Hewlett Packard Co., Santa Clara, CA, USA).

In vitro degradation studies were carried out in the Compact Anaerobic Workstation (DW Scientific, West Yorkshire; UK).

2.3. Synthesis of monomers

The synthesis of the monomers is given in Scheme 1.



Scheme 1. Synthesis of the monomers.

2.3.1. Synthesis of 4-[(4-hydroxyphenyl)azo]benzoic acid (HPABA)

13.2 g (96 mmol) of 4-aminobenzoic acid (ABA) was added to a mixture of 10 ml concentrated hydrochloric acid and 30 ml water, and cooled in an ice bath. While stirring, 50 g of ice and 15 ml of hydrochloric acid were added. Then, a solution of 7.2 g sodium nitrite in 14 ml of water was added at 0–5° C under stirring. After complete addition, the reaction mixture was stirred for a further 2 h. Then a solution of 13.2 g (140 mmol) of phenol in 10 ml of acetic acid was added and stirred for 30 min while maintaining the temperature below 5° C, followed by addition of 20 ml of a solution of 13.6 g of sodium acetate in 40 ml of water. Stirring was continued for 30 min. Thereafter, another 20 ml of sodium acetate solution was added and stirred for a further 30 min. The reaction mixture was left to warm to room temperature, after which 15 ml of a 20% solution of sodium hydroxide was added and left for 1 h at

room temperature. The precipitate was filtered and washed successively with water, 10% acetic acid in water and water again, then dried. It was then washed with chloroform, filtered and dried. A shiny dark-brown product was obtained.

Yield: 81%.

Melting point: the product decomposed at 252° C.

^{1}H-NMR in CD_3OD (δ in ppm): $\delta = 7.0$ (2H, *ortho* to -OH), $\delta = 8.0$ (4H, *ortho* to azo bond), $\delta = 8.25$ (2H, *ortho* to -COOH).

2.3.2. Synthesis of 4-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}benzoic acid (HEEEBA)

20.7 g (0.15 mol) of 4-hydroxybenzoic acid (HBA) and 28.6 g (0.17 mol) of 2-[2-(2-chloroethoxy)ethoxy]ethanol (CEEE) were dissolved in 225 ml of dry dimethylformamide in a 500 ml round-bottomed flask and 43.0 g of dry potassium carbonate was added. A reflux condenser attached with a calcium chloride tube was fitted to the flask. The mixture was refluxed at 160° C for 24 h, with constant stirring, then cooled, filtered and the excess of dimethylformamide was removed by vacuum evaporation. The residue was dissolved in chloroform, neutralised and the organic phase dried on magnesium sulphate. After filtration and evaporation of the solvent, the crude reaction product was purified by column chromatography (silica gel).

^{1}H-NMR in $CDCl_3$ (δ in ppm): $\delta = 7.8$–7.9 (2H, aromatic protons, *ortho* to -COOH), $\delta =$ 6.6–6.8 (2H, aromatic protons, *ortho* to -OCH_2-), $\delta = 4.2$–4.6 (2H, Ar-OCH_2-), $\delta = 3.5$–4.0 (10H, -CH_2OCH_2- and -CH_2OH).

Yield: 21.4%.

2.3.3. Synthesis of 4-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}phenylazobenzoic acid (HEEEPABA)

The same procedure as described for the synthesis of HEEEBA was followed using 7.26 g (0.03 mol) of HPABA and 5.57 g (0.033 mol) of CEEE.

After refluxing and cooling, the reaction mixture was added to diluted hydrochloric acid whereupon the product precipitated. It was filtered and washed with water, dried and washed successively with diethyl ether and chloroform. The brown reaction product has a melting point of 158–161° C.

Yield: 50.0%.

^{1}H-NMR in DMSO-d_6 (δ in ppm): $\delta = 3.43$–3.95 (10H, -CH_2OCH_2- and CH_2OH), $\delta = 4.13$–4.36 (2H, -CH_2OH), $\delta = 7.05$–7.20 (2H, aromatic protons, *ortho* to -OCH_2-), $\delta = 7.8$–8.05 (4H, aromatic protons, *ortho* to azo bond), $\delta = 8.13$–8.30 (2H, aromatic protons, *ortho* to -COOH).

2.4. Polymerization method

The monomers (10 mmol total concentration) were weighed in a round bottomed flask containing 1 mg of antimony trioxide as a catalyst. The

Table 1
Composition of the monomers in the poly(ether-ester) azo polymers

Polymer	HEEEPABA (mmol%)	HEEEBA (mmol%)	C_{16} (mmol%)	C_{12} (mmol%)	Yield (%)
PEE1	1.5	23.5	75.0		75.0
PEE2	1.5	23.5		75.0	63.1
PEE3	2.0	23.0	75.0		75.2
PEE4	2.0	23.0		75.0	72.1
PEE5	5.0	20.0	75.0		80.0
PEE6	5.0	20.0		75.0	64.3
PEE7	2.0	33.0	65.0		80.2
PEE8	5.0			95.0	58.1
PEE9	5.0		95.0		79.9

monomer mixture was heated at 205° C for 1 h under argon flow, and subsequently for 4 h under vacuum, with constant stirring.

The obtained polymers were cooled and dissolved in chloroform containing 1,1,1,3,3,3-hexafluoroisopropanol (five drops per 10 ml), and then precipitated in methanol, filtered and dried under vacuum. In this way terpolymers of HEEEPABA, HEEEBA, and 16-hydroxyhexadecanoic acid (HHDA) or 12-hydroxydodecanoic acid (HDA), and copolymers of HEEEPABA and HHDA or HDA were synthesized.

The monomer feed composition and the yield of the different azo polymers is given in Table 1.

2.5. Degradation studies

The cell-free extract was prepared from the cecal content of male Wistar rats, and was described previously (Van den Mooter et al., 1994).

Approx. 100 mg of the polymer was added to 10 ml of the cell-free extract and incubated under anaerobic conditions at 37° C, while stirring. After a specific period of time, the polymer was removed, thoroughly rinsed with water and subsequently dried under reduced pressure.

The dried polymer was dissolved in chloroform, containing a small amount (5 drops per 10 ml solution) of 1,1,1,3,3,3-hexafluoroisopropanol, and the absorbance was determined at 356 nm. The absorption peak of the azo polymers at 356 nm, due to the presence of the azo bonds, offers the possibility of performing the quantitative determination of the reduction of the azo groups. Data were collected after 24 and 48 h of incubation in the bacterial cell-free extract.

Since all absorption measurements were carried out in a range where linearity was observed between absorbance and concentration, absorption data were normalized by dividing them by the corresponding concentration (A/c). The A/c values were evaluated as a function of the incubation time.

The same procedure was repeated for polymers PEE2, PEE3, PEE4, PEE5, PEE6, and PEE8 after addition of 1% polysorbate 80 to improve the wettability of the polymers.

3. Results and discussion

Fig. 1 shows the decay of A/c of the polymers after incubation in the degradation medium for 48 h.

There was a consistent decrease of A/c as a function of time for all polymers. No significant difference was observed between the polymers PEE 1–PEE 7 (containing HEEEBA) ($p > 0.05$). Thus, the increase in HEEEBA concentration (from 20 to 33%) does not significantly affect the rate of degradation. Likewise, the rate of degradation is not significantly affected by substituting 12-hydroxydodecanoic (C12) acid for 16-hydroxyhexadecanoic acid (C16) in polymers containing HEEEBA.

A significant difference, however, was shown between polymers with HEEEBA and those without. The latter showed less degradation, and this is attributed to their hydrophobic nature. The incorporation of HEEEBA imparts hydrophilicity to the polymers due to increased hydrogen bonding. This makes them more accessible to azoreductase and hence they are degraded faster.

There was also a significant difference between polymers PEE8 and PEE9. Due to the same reason of hydrophobicity, PEE 8 is degraded to a greater extent than PEE 9. Structurally, the two polymers differ in that PEE8

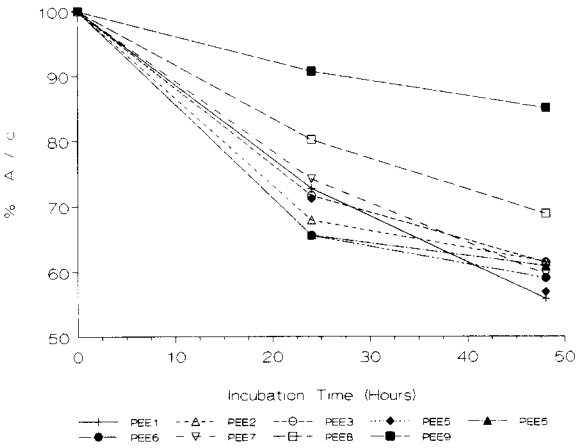


Fig. 1. Variation of A/c with time of poly(ether-ester) azopolymers.

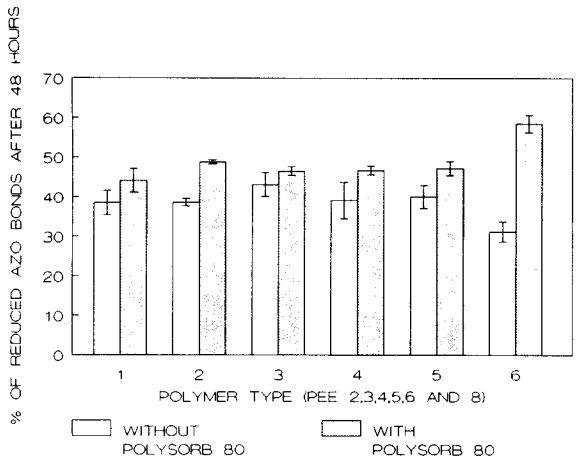


Fig. 2. Comparison of the degradation of poly(ether-ester) azopolymers with and without polysorbate 80.

contains dodecanoic acid (C12) and PEE9 has hexadecanoic acid (C16). The C16 being more hydrophobic compared to C12 makes PEE 9 less affected by azoreductase than PEE 8 due to reduced enzyme accessibility.

The addition of 1% polysorbate 80 prior to incubation of the polymers greatly increased the degradation process. Fig. 2 shows this effect for polymers PEE2, PEE3, PEE4, PEE5, PEE6, and PEE8. The increase in the rate of degradation after addition of polysorbate 80 further supports the above reasons given to explain the differences in the rate of degradation: the addition of polysorbate 80 increases the wettability and hence improves the contact between the polymers and the degradation medium. Thus, the polymers become more accessible to azoreductase resulting in a faster rate of degradation.

Biodegradation of azo polymers has been extensively studied in the literature. The degradation is said to be due to reduction of the azo bond(s) by azoreductase, a substrate non-specific enzyme produced by enteric bacteria (Gingell, 1973). The microbial susceptibility of azo bonds has stimulated scientists to investigate ways of utilizing polymeric azo carriers for physical targeting of drugs (Saffran et al., 1986; Kopecek and Kopeckova, 1992; Van den Mooter et al., 1992).

The mechanism by which the enzymatic azo bond reduction takes place has been investigated and postulated. Roxon et al. (1967) reported the reduction of the azo dye tartrazine by *Proteus vulgaris* from rats. They suggested that a soluble flavoprotein which is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) was involved. Gingell and Walker (1971) showed that soluble flavins can act under anaerobic conditions as electron shuttles between NADPH-linked flavoproteins and other electron acceptors. The azo compounds, being electron acceptors, can thus be reduced to primary amines.

While studying the reduction of azo dyes by anaerobic cultures of *P. vulgaris*, Dubin and Wright (1975) found that the rate of reduction was zero order with respect to dye. However, Wuhrmann et al. (1980) showed that the reduction of azo dyes by whole cultures and cell-free extracts of *Bacillus cereus* was first order with respect to substrate concentrations. Kopeckova and Kopecek (1990) related the rate of azo reduction to the ratio of substrate towards electron mediator concentrations. At higher electron mediator concentrations, compared to substrate concentration, the reduction process was zero order, the rate decreasing with increasing substrate concentration.

Addition of redox mediators (such as benzyl viologen) increased the rate of azo reduction (Chung et al., 1978). It has been hypothesized that the azo reduction is effected via both extracellular and intracellular enzymatic processes (Kopecek and Kopeckova, 1992).

4. Conclusion

The azo polymers containing HEEEBA are well degraded by azoreductase. The rate of degradation is further enhanced by the addition of a wetting agent, polysorbate 80. Therefore, these polymers have the potential to be used as matrices for colon-specific drug release. On the other hand, the polymers without HEEEBA demonstrate less degradation, due to hydrophobicity. Used as such, these polymers will not significantly release drugs embedded in their matrices. However, since the rate of degradation can be improved when a wetting agent is added, these

polymers are also recommended for further colon-specific drug release studies.

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