JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 597–601

Received October 19, 2006
Accepted December 11, 2006
DOI 10.1211/jpp.59.4.0016
ISSN 0022-3573

Iron (III) chelation and antioxidant properties of myo-inositol phosphorylated polymeric microspheres

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Abstract

New chelating and antioxidant polymeric microspheres were synthesized through precipitation polymerization of 4-*O*-(4-vinylbenzyl)-myo-inositol 1,3,5-orthoformate with ethylene glycol dimethacrylate and subsequent exhaustive phosphorylation of the myo-inositol groups with phosphoric acid. Three different microspheres using different molar ratios of the two co-monomers were prepared. The antioxidant properties of these polymers were evaluated in rat liver microsomal membranes. This study showed that the macromolecular systems are very effective at inhibiting iron-dependent lipid peroxidation of the membranes. This antioxidant effect is due to the chelating properties of polyphosphorylated inositol residues in the polymeric devices toward ferric ions. The amount of polyphosphorylated inositol in the microspheres was found to play a crucial role in determining the chelating efficiency of the polymers: the polymer with the greatest amount of polyphosphorylated inositol was the most powerful antioxidant polymer.

Introduction

Iron is an essential micronutrient required for every aspect of normal cell function, however iron overload is the cause of many degenerative processes because of the formation of free radicals via the Fenton reaction (Young & Woodside 2001; Turi et al 2004). To protect the human body from oxidative damage, many compounds, such as β-carotene, α-tocopherol, ferulic acid, phenols, lipoic acid, myo-inositol hexaphosphoric acid (phytic acid) and its derivatives (Boots et al 2002), generally known as antioxidants, are used (Cooper et al 2002). The protective mechanisms generally do not act independently but cooperatively in the form of a cascade (Halliwell et al 1995).

A compound can exert its antioxidant action by scavenging free radicals or by inhibiting their formation. Free radical generation is catalysed by transition metal ions, such as iron and copper, and so a compound can also exert antioxidant activity by chelating these metals (Pinchuk & Lichtenberg 2002).

In a healthy individual, iron levels are under extremely tight control and there is little opportunity for iron-catalysed free radical generating reactions to occur. In some cases, the iron status can change, either locally as in ischaemic tissue and hepatic and renal lesions (Kitamura et al 2005), or systematically as in genetic haemochromatosis, β-thalassaemia or transfusion-induced iron overload (Salo et al 2002). Iron chelating agents are very effective at preventing tissue injury due to iron overload (Liu & Hider 2002), on condition that all the six coordination sites of Fe^{3+} ions are occupied. If this condition is not met, free radical generation can occur (Liu & Hider 2002). Phytic acid is able to prevent the Fenton reaction because it occupies all the iron coordination sites (Graf & Eaton 1990). Not all the six phosphate groups are required for phytic acid activity. Myo-inositol tri-, tetra- and pentaphosphate are also effective (Spiers et al 1996; Phillippy & Graf 1997; Sala et al 2006). Recently, the possibility of inserting phytic acid derivatives into polymeric chains without losing the chelating ability was reported by Iemma et al (2005). In particular, a water-soluble antioxidant copolymer containing phosphorylated myo-inositol with activity comparable with phytic acid was successfully synthesized (Iemma et al 2005).

The aim of the present work was to evaluate the possibility of obtaining polymeric microspheres with chelating and antioxidant properties. These materials can be applied in

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Funding: This work was financially supported by MIUR (Programma di Ricerca di Rilevante Interesse Nazionale 2005) and University funds.

industrial and pharmaceutical fields when iron overload has to be removed from biological or environmental solutions (Sanchez et al 2001). For example, microspheres binding phosphorylated myo-inositol can be used in haemodialysis applications, in particular by introducing them in dialysis membranes; haemodialysis patients are exposed to oxidative stress, which contributes to cardiovascular disease and accelerated atherosclerosis, the major causes of mortality in these patients (Calo et al 2004).

The easy removal of iron from aqueous and physiological media represents the main advantages of these materials compared with soluble compounds. Moreover, the polymeric microspheres that we propose are easy to synthesize and handle. The synthetic strategy involved the precipitation polymerization of functional monomer 4-*O*-(4-vinylbenzyl)-myo-inositol 1,3,5-orthoformate with the crosslinking agent ethylene glycol dimethacrylate. The matrices were subsequently phosphorylated using phosphoric acid to give the final microspheres. Both the non-phosphorylated and the phosphorylated polymers were characterized by scanning electron microscopy and dimensional analysis. The chelating properties of the phosphorylated microspheres were tested in rat liver microsomal membranes previously exposed to iron overload.

Materials and Methods

Materials

Reagents and solvents were obtained from Sigma-Aldrich (St Louis, MO, USA). All solvents were reagent grade or high-performance liquid chromatography grade.

Polymer preparation

4-*O*-(4-Vinylbenzyl)-myo-inositol 1,3,5-orthoformate (1.96 mmol for polymer I; 1.63 mmol for polymer II and 1.37 mmol for polymer III) and ethylene glycol dimethacrylate (5.88 mmol for polymer I; 6.52 mmol for polymer II and 6.85 mmol for polymer III) were dissolved in acetonitrile (55 mL) in a 100-mL round-bottomed flask and then 100 mg of azobis-isobutyronitrile was added. The polymerization mixture was degassed by purging with nitrogen for 10 min at 0°C. The flask was then gently agitated (40 rev min^{-1}) at 60°C for 24 h. The particles were filtered, washed with 100 mL of 2-propanol, methanol and acetone and finally dried under vacuum overnight at 40°C.

Phosphorylation of myo-inositol residues

Polymers (500 mg) were added to 50 mL phosphoric acid (85 wt%) and heated at 120°C for 12 h. After cooling, the particles were filtered, washed with distilled water to pH 7.0 and then with 100 mL of 2-propanol, methanol and acetone. Particles were successively dried under vacuum overnight at 40°C.

Particle size analysis

Scanning electron microscopy photographs were obtained with a Jeol JSMT 300 A; the surface of the samples was made conductive by depositing a gold layer on the samples in a vacuum chamber.

The mean particle size and size distribution of the microspheres were measured using an image processing and analysis system, a Leica DMRB equipped with a LEICA Wild 3D stereomicroscope.

Approximately 10000 particles were counted for each preparation and each measurement was the mean of three samples.

Analysis of phosphate groups

Ammonium molybdate (10 g) and ammonium vanadate (0.50 g) were dissolved in HNO_3 (70 mL, 63% v/v) and distilled water (430 mL). In 100-mL volumetric flasks, five solutions were prepared by introducing 25 mL of the vanado-molybdate reagent and 13, 15, 27, 19, 21 mL, respectively, of a KH_2PO_4 solution with a concentration corresponding to a 200 mg L^{-1} solution of P_2O_5; distilled water was added to 100 mL. The solutions were analysed using a UV-Vis spectrophotometer ($\lambda = 420$ nm) in order to obtain a calibration curve. Each polymer (100 mg) was added to a solution of 5 mL distilled water, 5 mL HCl (12 M) and 5 mL HNO_3 (14.5 M) and heated at 100°C for 15 h. After cooling, the particles were filtered and the solutions were added in a 100-mL volumetric flask to 25 mL of the vanado-molybdate reagent; distilled water was added to 100 mL. The solutions were analysed using a UV-Vis Jasco spectrophotometer V-530 at 25°C ($\lambda = 420$ nm). Each measurement was the mean of five samples.

Malondialdehyde (MDA) assay

Aliquots of polymers over the range 0.5–6 mg mL^{-1} were added to the microsomes, gently suspended by a Dounce homogenizer and incubated at 37°C in a shaking bath, under air, in the dark, in the presence of 30 μM Fe^{3+} and 300 μM ascorbate solutions. Aliquots of 1 mL of microsomal suspension were analysed as reported by Carlson et al (2006) to evaluate the antioxidant effect of the polymers.

Statistical analysis

Results are expressed as the mean ± s.e.m. Multifactorial two-way analysis of variance was adopted to assess any difference among treatments and times. When the *F*-tests were significant ($P < 0.05$), post-hoc comparisons of means were made using Tukey's honestly significant difference test. Data in Table 1 were analysed using one-way analysis of variance. When significant values were found ($P < 0.05$), post-hoc comparison of means was made using the Fisher's test.

Results and Discussion

Synthesis and characterization of polymers

To prepare antioxidant polymeric microspheres we used myo-inositol orthoformate monomer, which can be obtained from commercial myo-inositol by a flexible procedure

Table 1 Mean particle size, polydispersity, phosphate content, malondialdehyde (MDA) inhibition and IC50 of polymers

Polymer	Mean particle size (polydispersity)		Phosphate content (mols of phosphate $\times 10^{-7}$ (mg polymer)$^{-1}$)	MDA inhibition (%)					IC50 (mg mL^{-1})
				Polymer concentration (mg mL^{-1})					
	Non-phosphorylated polymers	Phosphorylated polymers		0.5	1	2	3	6	
I	4.69 (1.01)	4.76 (1.02)	7.20 ± 0.26	40 ± 5^{a}	67 ± 4^{b}	87 ± 3^{c}	93 ± 2^{c}	96 ± 2^{c}	0.70 ± 0.03
II	5.39 (1.01)	5.15 (1.04)	5.29 ± 0.15	35 ± 3^{a}	59 ± 3^{b}	80 ± 3^{c}	89 ± 5^{d}	93 ± 3^{d}	0.82 ± 0.02
III	4.91 (1.03)	5.03 (1.04)	4.70 ± 0.18	18 ± 4^{a}	41 ± 5^{b}	70 ± 3^{c}	76 ± 3^{c}	81 ± 4^{c}	1.30 ± 0.04

Results of MDA inhibition (% inhibition after 30 min incubation at 37°C) are the mean ± s.e.m. of six separate experiments. $^{a–d}$Within the same treatment, values not sharing a letter were significantly different ($P < 0.05$; Fisher's test).

reported in the literature (Baudin et al 1988; Vacca et al 1989; Hawkins et al 1993; Flores-Mosquera et al 1998). This monomer was used to prepare the spherical antioxidants polymers (Table 1). The spherical shape was chosen because of the homogeneous distribution of the chemical group on its surface (Ye & Mosbach 2001; Puoci et al 2004), and precipitation polymerization was chosen as the synthetic procedure (Wang et al 2003). The proposed mechanism was characterized by two parts: nucleation and growth of microspheres. The reaction begins as a usual solution polymerization. The monomers and initiator were dissolved in the organic solvent and during the polymerization oligomers are formed. After a certain period of time, the concentration of oligomers becomes sufficiently high to allow radical polymerization of oligomers to form a microgel (nucleation). Each seed (microgel) then grows by continuous capture of oligomers. This prevents the occurrence of any further nucleation and hence uniformly sized particles are produced. During polymerization the growing polymer chains are separated from the continuous medium by enthalpic precipitation in cases of unfavourable polymer–solvent interactions, or entropic precipitation in cases where crosslinking prevents the polymer and the solvent from freely mixing.

Myo-inositol residue phosphorylation was carried out in phosphoric acid to obtain the final particles (Figure 1). Generally, the deprotection of the hydroxy groups of myo-inositol is carried out in a trifluoroacetic acid solution (Sarmah & Shashidhar 2003). In our experiments, microspheres submitted to phosphorylation with and without trifluoroacetic acid treatment showed the same degree of phosphorylation (data not shown). Thus, with the acidic conditions of the phosphorylation step, it was possible to obtain at the same time the cleavage of the orthoformate and the phosphorylation of all the hydroxy groups.

The spherical geometry and the practically monodispersion of the prepared samples were confirmed by scanning

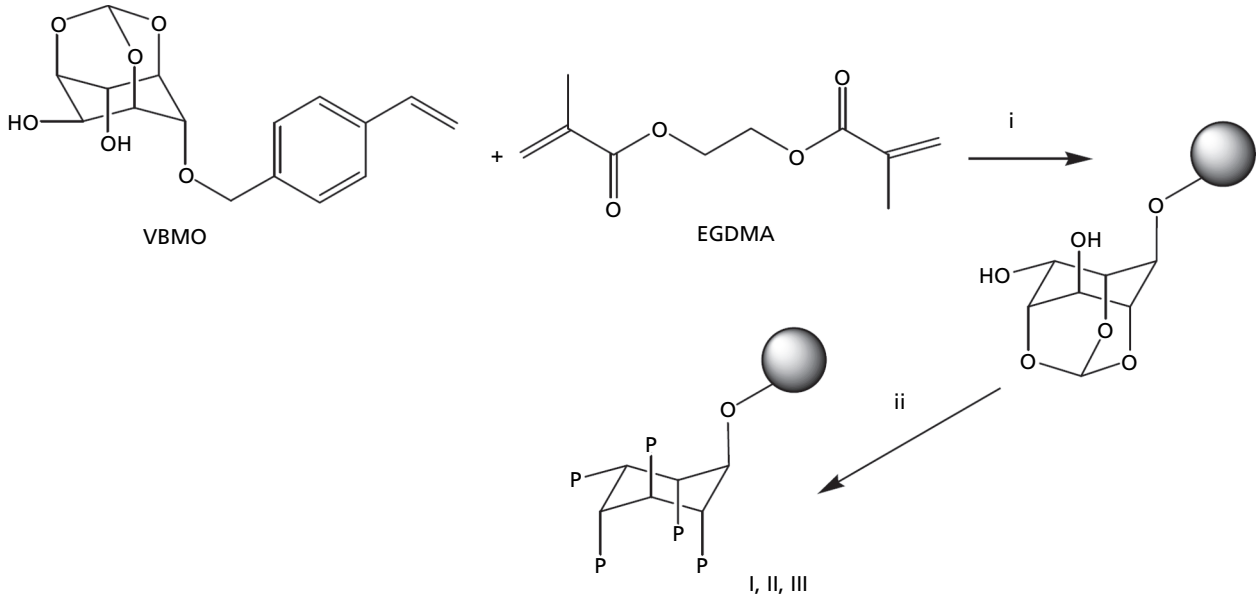

Figure 1 Synthesis of polymers I, II, III. VBMO, 4-*O*-(4-vinylbenzyl)-myo-inositol 1,3,5-orthoformate; EGDMA, ethylene glycol dimethacrylate; P, phosphate group. Reagents and conditions: i: azobisisobutyronitrile, CH_3CN, 60°C, 24 h; ii: H_3PO_4, 120°C, 12 h.

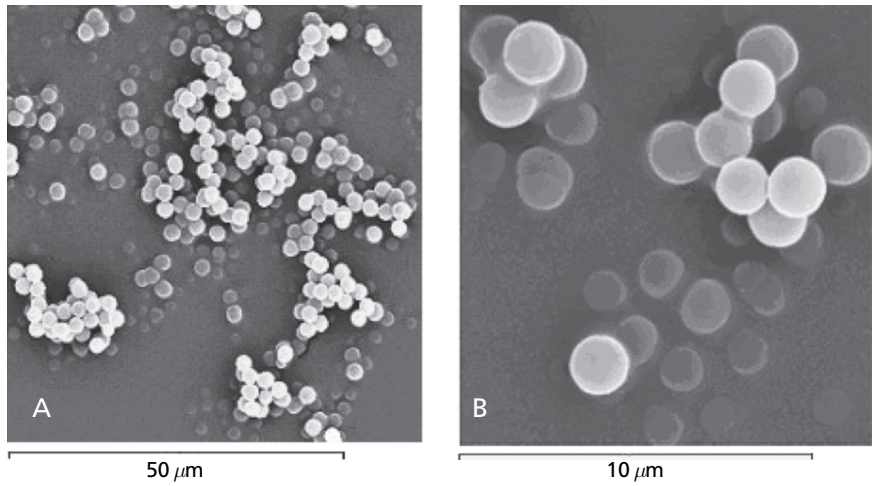

Figure 2 Scanning electron micrographs of non-phosphorylated (A) and phosphorylated (B) polymer I.

electron micrographs (Figure 2) and dimensional analysis (Table 1). The mean particle size and the polydispersity of the microspheres are shown in Table 1.

In the scanning electron micrographs (Figure 2) of microparticles it was possible to observe that the morphologic characteristics of the prepared samples did not change. Dimensional analysis showed that the phosphorylation did not interfere significantly with the dimensions and polydispersity values of the particles (Table 1).

Microparticles were also characterized by quantitative determination of the phosphate amount in order to determine the functionalization degree (Table 1). This procedure (Decreto Ministeriale 1986; Munoz et al 1997; Morais et al 2004) involved the hydrolysis of the phosphate groups from the polymeric backbone and, subsequently, the reaction of freed phosphates with a vanado-molybdate reagent.

Chelating properties of the polymers

The chelating properties of the microspheres was examined in rat liver microsomal membranes (Ohta et al 1997). The membranes were exposed to oxidative stress with an iron overload, in particular a Fe^{3+}/ascorbate induced lipid peroxidation was performed. It is known that Fe^{2+}, produced by redox reaction between Fe^{3+} and ascorbate, develops radical species via the Fenton reaction (Minihane & Rimbach 2002). The radicals so developed can react with the fatty acids of the microsomes producing many toxic compounds such as MDA. The MDA amount is related to the pro-oxidant efficiency of the Fe^{3+}/ascorbate system and therefore to the iron content. Thus, by employing an iron chelating agent, reductions in both iron pro-oxidant efficiency and MDA concentration were observed. In our study, the MDA amount in the oxidized membranes was evaluated by measuring the concentration of MDA–thiobarbituric acid adduct (Tatum et al 1990). Polymers did not interfere with the MDA assay as no MDA was detected in the medium containing the antioxidants alone in the absence of the microsomes.

Microspheres were added to rat liver microsomal membranes exposed to iron overload at concentrations over the range of 0.5–6 mg mL^{-1} (Table 1). The three polymers were found to be strong antioxidants, protecting the membranes from Fe^{3+}/ascorbate-induced lipid peroxidation, and the IC50 values of each polymer clearly showed that the most phosphorylated polymer was also the most powerful, in agreement with the theory that the antioxidant properties depend on the amount of phosphorylated myo-inositol in the matrices. As seen in Figure 3, the antioxidant effects of phosphorylated polymer I was also dose- and time-dependent. Phosphorylated polymers II and III had a similar but lesser effect on MDA formation (data not shown).

Conclusion

New antioxidant and iron-chelating polymeric microspheres were successfully synthesized using a two-step procedure. The first step was the precipitation polymerization of myo-inositol

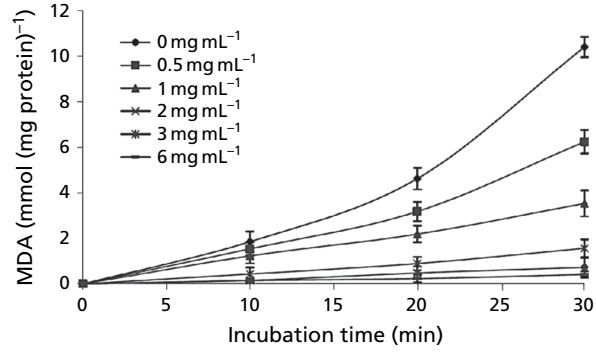

Figure 3 Effects of polymer I on malondialdehyde (MDA) production as a function of incubation time. Results represent the mean ± s.e.m. of six separate experiments.

derivatives in the presence of a cross-linker agent. The second step was the exhaustive phosphorylation of the myo-inositol derivatives. The particle morphology indicated a spherical shape and a micrometric and monodisperse size distribution. Microspheres maintain the iron chelating properties of polyphosphorylated myo-inositol and were tested in rat liver microsomal membranes. The polymers were found to be potent antioxidants, acting through the inhibition of iron-catalysed free radical generation in the membranes. This ability was related to the myo-inositol content and confirms the hypothesis that this polymeric device could be useful in preventing oxidative damage due to iron overload. Furthermore, this study could be the starting point for the realization of a system to be applied when an iron overload has to be removed from physiological or environmental media.

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