Macromol. Symp. ***144**, 413–422 (1999)*

Modification of chitosan for construction of efficient antioxidant biodegradable macromolecular systems

V.A.Alexandrova,* G.V.Obukhova,* N.S.Domnina,** D.A.Topchiev*
*Topchiev Institute of Petrochemical Synthesis RAS, Leninsky pr. 29, Moscow, 117912, Russia
**Chemistry department of St.-Petersburg University, Universitetskii pr.2, Peterhof, St.- Petersburg, ,198904, Russia

Summary: The chitosan derivatives containing antiradical fragments in the polymer side chain have been synthesized by interaction of the partially quaternized chitosan(QCH) with gallic acid (GA). The antioxidative activity of the chitosan derivatives - QCH-GA was investigated by thiobarbituric method. Introduction of GA fragment in amount of 2. 0 mass % in the structure of QCH resulted in appearance of pronounced antioxidative activity of the polymeric system contrary to initial chitosan for which this activity was equal to zero. It was found that QCH-GA was a markedly higher effective inhibitor in a peroxidase - catalyzed oxidation of the model amine than the low-molecular antioxidant - GA. Targetted chitosan modification resulted in a substantial raize of the polymeric antimutagenic (at γ-irradiation) efficiency, which for QCH-GA was equal to 92 % in comparison with the protective effect of the initial chitosan - 42 % (plant test-system, barley seeds, γ = 15 Gr).

Introduction

Chitosan, a natural biodegradable polysaccharide , is now well- known for its very interesting biological properties such as antitumor, haemo- and immunostimulative ones and so on. The other important factor making chitosan a leading candidate in many applications is the large variety of useful phisical forms: powders, films, fibers, sponges and the others. The unique features of mechanical and chemical properties of these materials offer numerous largely unexplored applications in technology, chemistry, medicine and agriculture1). Various types of gels, membranes and fibers, including polycationic and water-soluble materials, can be formed2,3) .

One of the most important problems in polymer chemistry and technology is stabilization of a polymer material. From this point of view, development of various approaches for creation of polymeric systems being possessed of antioxidative properties on the basis of chitosan is very perspective for possible application in various spheres of human activity.

For this purpose we intended to modificate the chitosan macromolecule by introducing GA derivatives- effective inhibitors of radical reactions into the polymer side chain. It is

CCC 1022-1360/99/$ 17.50+.50/0

known also that the GA moiety enchances the antioxidative activity of various natural antioxidants, for example, green tea polyphenols [4]. As we supposed such a modifcation at the same time would help us to overcome the main drawbacks of these low- molecular inhibitors, particularly, an ability to exhibit properties of both anti- and prooxidants in dependence on the conditions[5]. The dual character of action of these compounds is connected with the formation of radical and molecular products at their oxidation. We proposed to stabilize GA derivatives by their introduction into a polycationic matrix.

Materials and methods

Materials and synthesis

The copolymer of N,N- diallyl-N,N- dimethylammonium chloride and acrylic acid DADMAC: AA (composition 30: 70) was synthesized on the procedure developed at the Petrochemical Synthesis Institute RAS. The samples of the DADMAC: AA copolymer were thoroughtly purified according to the known method [6]. The purity of the substances was controlled using the data of ^{13}C NMR spectra obtained using MSL-300 spectrophotometer (Bruker,Germany). The ternary copolymers of diallyldimethylammonium type- (TCP-GA) were synthesized by interaction of DADMAC:AA copolymer with gallic acid hydrazide using CCD (Fluka) as a condensing agent according to the procedure developed by the authors [7]. The purity of polymeric products was cheked up by GPC on Sefadex- LH-20 column (a solvent - water or methanol). Chitosan- commercical material (Russia) from shell chitin (85% of deacethylation) was used as an initial polymeric matrix for synthesis. Partially quaternized chitosan (QCH) was obtained by interaction of chitosan with MeJ in the presence of $(Et)_3N$ [8]. The chitosan derivatives- QCH-GA were obtained by interaction of the partially quaternized chitosan with GA using CCD (Fluka) for preliminary activation of GA according to the procedure [9]. The samples of QCH-GA were isolated by a precipitation in aceton with reprecipitation from water in aceton to remove the low- molecular impurities completely (control of the eluent by UV spectroscopy). The quantity of the covalent bound GA fragments in QCH-GA was determinated by UV spectroscopy, the absorbtion maximum at $\lambda = 268$ nm on Spectrophotometer SF-56 ("LOMO", Russia).

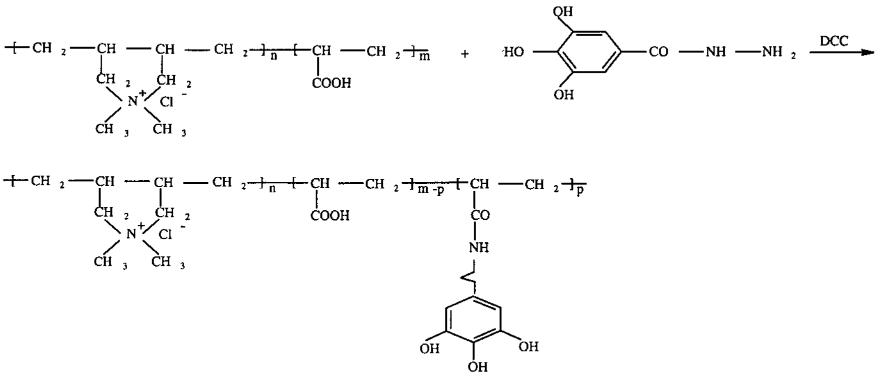
Methods

The antioxidative activity of antioxidants was determined according to the technique[9] with the Twin -80 [poly(oxyethylenesorbitan monooleate)] based on oxidation by air oxygen in the presence of a ferrous salt and ascorbic acid in aqueous solutions, followed by photometric determination (CF -56 spectrophotometer) of the amount of malonic aldehyde produced. The antioxidative activity AOA of polymeric antioxidants was characterized by a decrease in the intensity of absorption of the complex formed between malonic aldehyde with 2- thiobarbituric acid at $\lambda = 532$ nm ($\varepsilon = 1.56 \times 10^{-5}$ l/ (mol cm) in solutions containing antioxidant in comparision to the control experiments according to the equation AOA = 100- (a/b) × 100, % , where a and b are the amounts of malonic aldehyde formed in the solution and in the control experiments, respectively. Twin-80 (Ferak) with $M = 1.3 \times 10^3$ was used.

The inhibitory activity of QCH- GA- 2 was determined according to the method based on an oxidation of 4,4'- diamino-3,3'- dimetoxybiphenyl (DA) by the complex of horseradish peroxidase with hydrogen peroxide comparing the results obtained for polymeric and low molecuar antioxidants. Horseradish peroxidase (PH) ("BILAR",Latvia) was used. The concentration of PH was determined photometrically (CF-56 spectrophotometer). The experimental procedure is described in detail [10]. The antimutagenic activity of TCP-GA and QCH-GA was determined using the known method [7].

Morphology

To verify our suggestion on possible stabilization of GA derivatives by their incorporation into the polycation matrix we used as a model a synthetic polycation - a copolymer of N,N- diallyl-N,N- dimethylammonium chloride and acrylic acid DADMAC:AA (composition 30 :70). It was shown in our previous investigations that this copolymer had a positive charge in solution at pH= 6.8. The selection of this copolymer as a polymeric base was grounded on the ability of such polycations to scavenge short-lived water radicals (such as hydroxy radical $OH^{\bullet}$) at radiolysis as it was shown by ESR method in our previous investigation[11]. Ternary (TCP-GA) copolymers differing in the proportion of modified acrylic acid units were synthesized by covalent binding of the antiradical fragment with the polycation through an amide bond formation with carbodiimide (CCD) as a condensing agent:



The quantity of the covalent bound GA- derivative in ternary copolymers (1.1 and 2.4 mass %) was determined by UV- spectroscopy. The introduction of GA fragments in such a relatively low quantity was grounded on the high scavenger ability of GA derivatives in radical reactions. Besides this, as it had been shown in our previous investigation an incorporation of a relatively high quantity (more than 5 mol %) of the hindered phenol in the polymer structure was not favorable for an achievement of both antiradical and antimutagenic high efficiency and at the same time maintaining low toxicity of the polymer[12)].

The antioxidative activity of the ternary copolymers obtained was determined by the technique based on the oxidation of unsaturated fatty-acids by air oxygen in the presence of a ferrous salt and ascorbic acid in aqueous solutions, followed by a photomeric determination of the amount of the malonic aldehyde produced. The data of the Table 1 show that TCP-GA exhibit a pronounced antioxidative activity, contrary to the GA derivative, for which the evident prooxidative activity was observed.

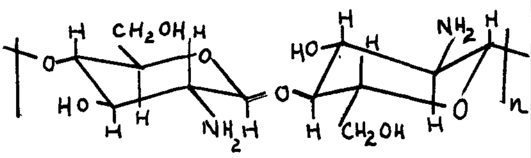
Table 1. ANTIOXIDATIVE ACTIVITY OF GALLIC ACID DERIVATIVES AND POLYCATIONS OF DIALLYLDIMETHYLAMMONIUM TYPE

N	SUBSTANCE	ANTIOXID. ACTIVITY, % CONTROL.
1	ME GA	- 19
2	CP DADMAC : AA 30 : 70	17
3	TCP-GA GA CONTENT, MASS. %	
	1,1	46
	2,4	44

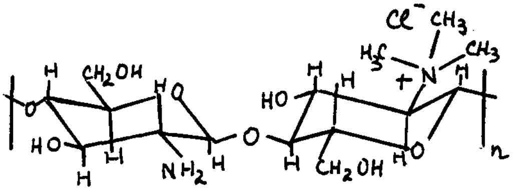
Besides this, the introduction of GA fragments in amount of 1.1 and 2.4 mass % in the copolymer structure results in a substantial increase of an antimutagenic effect (91 %) under γ-irradiation in comparison with the protective effect of the initial copolymer (52 %) (plant test-system- barley seeds, γ= 15 Gr).

Thus, combination of a polycationic nature of the polymeric matrix and the low quantity of the structural fragment of gallic acid in the polymer side chain allows to create the polymeric systems with both substantial antioxidative and antimutagenic activity.

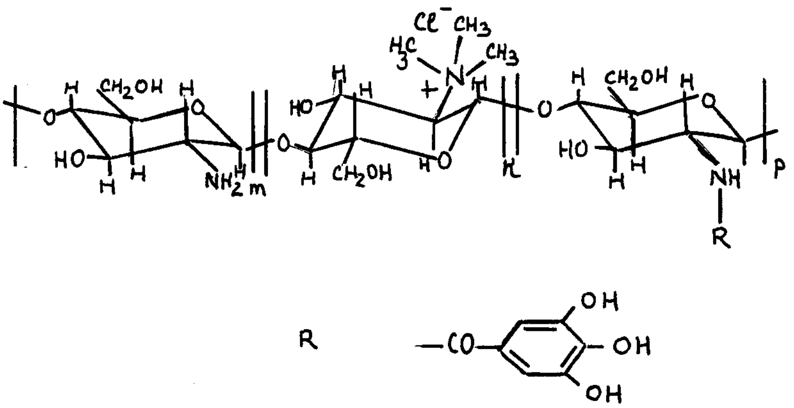
Taking into account this consideration we tried to obtain an efficient biodegradable antioxidative system on the chitosan basis introducing a relatively low quantity of GA fragments in the structure of partially quaternized chitosan. For this purpose chitosan, in which 40% of primary amine groups had been converted into the quaternized ammonium groups (to increase the positive charge of the macroion) was chosen as an initial polymer. The antiradical fragments were incorporated into the polymer side chain by covalent binding through amide bond formation using carbodiimide for preliminary activation of GA. Water soluble polyantioxdants - QCH-GA with a low content of GA fragments- 0.5 and 2.0 mass % in a macromolecule of the chitosan derivative were synthesized:



CHITOSAN-CH



QUATERNIZED CHITOSAN-QCH



QCH-GA

The antioxidantive activity of the polymeric polyphenols was determined using the TBA-test mentioned above . As we can see from the data given in the Table 2 , the partial chitosan quaternization and incorporation of GA fragments in amount of 2.0 mass % in the macromolecular structure result in appearance of pronounced antioxidative activity of the polymeric system, contrary to initial chitosan, for which in the case of this test the activity is equal to zero.

Table 2. ANTIOXIDATIVE ACTIVITY OF THE CHITOSAN DERIVATIVE AND GALLIC ACID

N	SUBSTANCE	ANTIOXID. ACTIVITY, % CONTROL.
1	ME GA	- 20
2	CH	0
3	QCH-GA- 2	48

It should be noted that TBA-test is used most frequently for measuring of the peroxidation level according to the final oxidation products in fatty acids, food stuffs and membranes. Phenolic antioxidants being good electron donors are ideal agents for an interaction with peroxy radicals:

$$\mathrm{Ar\,OH} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{Ar\,OH}^{+} + \mathrm{ROO}^{-}$$

$$K \cong 10^{6}\,\mathrm{m}^{-1}\mathrm{s}^{-1}$$

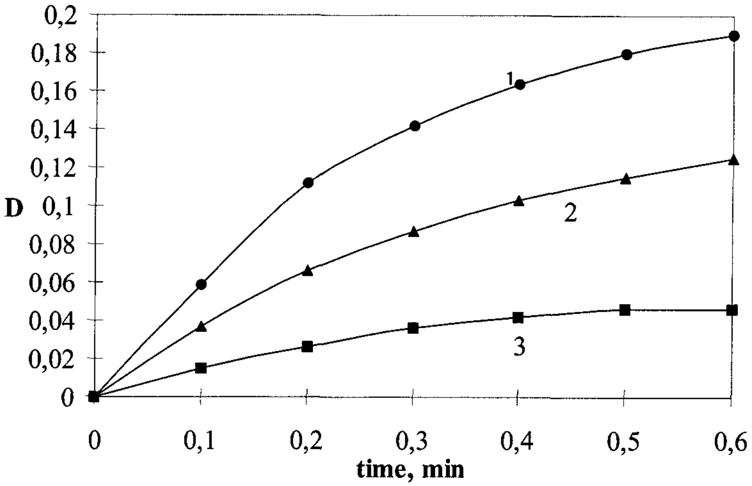
followed by a rapid deprotonation:

$$\mathrm{ArOH}^{+} \rightarrow \mathrm{ArO}^{\bullet} + \mathrm{H}^{+}$$

However, to evaluate the antioxidative properties of the polymeric antioxidant -QCH (GA) in the " high rate" oxidation processes which proceed under an oxidative stress (as at γ-irradiation) we used another test-system.

The complex of horseradish peroxidase with hydrogen peroxide oxidizes substrates very quickly according to a radical unielectron mechanism$^{10)}$. As we can see from the data of the Fig.1 the polymeric gallic acid derivative is a markedly higher effective inhibitor in peroxidase- catalyzed oxidation of a model amine (the lower curve) than the low-molecular antioxidant - GA (the middle curve) with respect to oxidation of the amine without any inhibitor- a blank test (the upper curve).

Fig.1 Inhibitory activity of polymeric and low- molecular antioxidants in peroxidase -catalyzed oxidation of the diamine



where 1- a blank test - oxidation of DA without inhibitor
2- oxidation of DA, inhibitor GA (0,01 mmol/l)
3- oxidation of DA , inhibitor QCH-GA-2 (0,01 mmol/l)

The inhibition constants of the peroxidase - catalyzed amine oxidation by polymeric and low-molecular antioxidants calculated on the kinetic data and the values are given in the Table 3. The inhibition constants of polymeric and low-molecule antioxidants in peroxidase-catalyzed oxidation of DA.

Table 3. **The inhibition constants of polymeric and low- molecular antioxidants in peroxidase-catalyzed oxidation of DA.**

N	INHIBITOR	K, 1/ MOL.S
1	GA	1.1×10^4
2	QCH-GA-2	4.6×10^4

Comparing the results obtained one can see that the value of the constant for the polymeric antioxidant is four times larger than that for a gallic acid. The data obtained confirm that incorporation of GA fragments in amount of 2.0 mass.% in the structure of the partial quaternized chitosan results in appearance of the pronounced antioxidative activity of the polymeric system.

The antimutagenic activity of these chitosan derivatives at gamma-irradiation has been investigated using a plant test-system (barley seeds). The data obtained confirm that the targetted modification of the chitosan matrix results in a rise of the antimutagenic efficiency of the polymeric system up to 92 % in comparison with the protective effect of the initial chitosan- 42 % (plant test-system- barley seeds, γ=15 Gr).

Conclusions

Thus, the strategy for the construction of efficient antioxidative biodegradable macromolecular systems on the chitosan basis has been developed. Such inhibitors can be used in protection against oxidative damage under normal conditions and under condition of an oxidative stress.

This work is supported by Russian Foundation for Basic Reseach(project 97-03-32773).

References

1) M.G. Peter, *J. Macromol. Sci. App. Chem.*, A. **32** (4), (1995), p.629-640

2) K.D. Yao, Y.S. Yin, M.X. Xu, Y.F. Wang, *Polym. Int.*, **38** (1), 1995,p.77-248

3) T.Y. Hsien, G.L.Rorrer, *J. Chem. Technol. Biotechnol.*, Jul.,**63**(3),(1995), p. 247-248

4) Zhi Y. Wang, Shu J. Cheng, Zong C. Zhou, Mohammad Athar, Wasiuddin A. Khan, David R. Bickers, Hasan Mukhtar, *Mutation Research* ,**223**, (1989), p. 273-285

5) S.P. Odintzova, K.E. Krugljakova, *Dokl. Akad. Nauk SSSR*, v. **226**, № 2,(1976),p.456-459

3)N.M. Boyarkina, V.V. Kryuchkov, E.S. Parkhamovich, L.A. Amburh, D.A. Topchiev, V.A.Kabanov, *Plasticheskiye Massy*, **8**, (1987), p.17-20

7) V.A. Shevchenko, D.A.Topchiev, V.A.Alexandrova, E.B.Kotlyarova, A.P. Odin, N.S.Domnina, *RU Pat.* **N 2043368.**

8)L.A.Nud'ga, E.A. Plisko, S.N. Danilov, *Zhurnal Obshchey Khimii*,

43 (12), (1973),p. 2756-2760

9) V.A.Alexandrova, D.V. Ryzhkov, V.A., V.A. Shevchenko, D.A.Topchiev, N.C.Domnina,E.B. Kotlyarova, G.V. Obukhova, *RU Patent* № **2101919**

10) E.I.Karasyova, Yu.P.Losev, D.I.Metelitza, *Biokhimiya*, v.**62** (10),(1997),p.1256-1263

11)V.A.Alexandrova, V.N. Belevskii A.P.Odin, D.A.Topchiev, *Dokl.Akad. Nauk RAS*,v.**348**,№ 1,(1996),p.51-56

12) V.A.Alexandrova, E.B.Kotlyarova, A.P. Odin, N.S.Domnina, V.A. Shevchenko, D.A.Topchiev, *Radiat.Biol.Radioekologiya.*,v.**35**,№ 5,(1995),p. 746-752