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Radical scavenging activity of 5-methylpyrrolidinone chitosan and dibutyryl chitin

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ABSTRACT

This study compares the behaviour of 5-methylpyrrolidinone chitosan (MPC), a substituted hydrophilic polymer bearing free –OH and $–NH_2$ groups, and dibutyryl chitin (DBC), a novel lipophilic *O*- and *N*-persubstituted chitin, toward free radicals. By means of EPR measurements their capacity of trapping radicals photochemically generated from PTOC esters was assessed. The fate of radicals inside the polysaccharides was checked on the basis of the structure of products obtained after photolysis and characterized by NMR spectroscopy. The experimental data clearly demonstrated that hydrophilic chitosans and persubstituted chitins possess remarkable radical blocking activity even though the nature of the process should be rather different between MPC and DBC. The DBC substrate hosts the radicals in protected and poorly reactive environment, whereas hydrophilic chitosan substrates favor a more rapid annealing of the radicals. MPC takes part in radical reactions with a consequent likely fragmentation of the polymer chain. A photochemical experiment carried out on chitosan confirmed the behaviour observed for MPC.

1. Introduction

During the last few years, some articles were published on the anti-oxidative and radical scavenging activities of chitosans. Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging. Superoxide anion, hydroxyl radical, and hydrogen peroxide generated by metabolic processes or from exogenous factors and agents initiate the peroxidation of membrane lipids, leading to lipid peroxides (Xing et al., 2004). Re-acetylated water-soluble chitosans inhibited thiobarbituric acid reactive substances formation in peroxide-induced lipid peroxidation (Matsugo et al., 1998). Water-soluble maleic acid-grafted hydroxypropyl chitosan and carboxymethyl chitosan, showed scavenging activity against hydroxyl radical (Xie, Xu, & Liu, 2001).

Preferred for their water-solubility, the oligomers did not form their own radicals, as a point of difference from ascorbic acid that forms the ascorbyl radical. The free amino group at the C-2 position plays a major role together with the primary alcohol group in the scavenging activity. Chitosan itself and chitosan oligomers were reported to scavenge 1,1-diphenyl-2-picrylhydrazyl, hydroxyl, carbon-centered and superoxide radicals (Park, Je, & Kim, 2004). On the other hand, *N*-acetylglucosamine (10 g/l) showed no activity against all tested radicals.

Another route to circumvent the difficulty represented by the poor solubility of chitosan is the derivatization into the fully water-soluble O^6-aminoethyl chitosan: Je and Kim (2006a) suggested that the additional amino group enhanced the scavenging activity that was as high as 60% for concentrations of 600 μg/ml, with human lung fibroblast viability of ca. 92%. They further described the potential of DEAE–chitosan to suppress the growth of tumor cells (Je, Cho, & Kim, 2006b). The results obtained by Lin and Chou (2004) for water-soluble chitosans *N*-alkylated with cellobiose, maltose and lactose units are in line with the previous ones in so far as the highest scavenging activity was observed for rarely substituted products (d.s. 0.20–0.30) indicative of the main role of the primary amino group. At 400 μg/ml they exhibited >60% scavenging activity for hydrogen peroxide.

Schiff bases obtained from chitosan do not exhibit scavenging activity (Guo et al., 2005) as a point of difference from quaternary chitosan (Guo, Liu, Chen, Ji, & Li, 2007). Highly sulfated partly depolymerized chitosans were also reported to be effective against superoxide and hydroxyl radicals. Gold–chitosan nanocomposites depress the activity of hydroxyl radicals (Esumi, Takei, & Yoshimura, 2003).

High MW *O*-carboxymethyl chitosan at the maximum concentration of 40 mg/ml did not show any scavenging activity against the superoxide anion, but low MW *O*-carboxymethyl chitosan (1100–4350 Da) showed very modest activity, indicative of the importance of the molecular size (Sun, Xu, Liu, Xue, & Xie, 2003). In fact the *O*-carboxymethyl group is unfavorable because

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it drastically lowers the number of primary alcohol functions necessary for the scavenging action. Nevertheless, carboxymethylated chitosan (no CM position specified) was used with the intention of protecting the chondrocytes from induced apoptosis: in fact it restored the level of mitochondrial membrane potential, down-regulated the NO synthase expression and scavenged reactive oxygen species in chondrocytes (Chen, Liu, Du, Peng, & Sun, 2006).

Carboxyl and quaternary amino groups were introduced into chitosan oligomers with different degrees of substitution for the purpose of altering the amount of hydrogen atoms capable of reacting with radicals, and modifying the chelating capacity. In fact the scavenging of 1,1-diphenyl-2-picrylhydrazyl and carbon-centered radicals was directly dependent on the amount of abstractable hydrogen atoms in the chitosans, while the chelation of Fe(II) contributed to the hydroxyl radical scavering as a separate process (Huang, Rajapakse, & Kim, 2006).

While chitin is notoriously insoluble in aqueous and organic media, with a few exceptions, carboxymethyl chitin is an example of a water-soluble derivative, while dibutyryl chitin is a highly esterified, fully biocompatible derivative soluble in a variety of organic solvents (Muzzarelli et al., 2005) and it is an interesting model containing available hydrogen atoms.

Scope of the present research work is therefore the definition of the radical scavenging properties of chitosan, the plain cationic polysaccharide, Methylpyrrolidinone chitosan (MPC), a water-soluble amply tested biomaterial suitable for biomedical applications, and dibutyrylchitin (DBC), a chitin derivative unique for its solubility in organic solvents, in order to verify the role of abstractable hydrogen atoms. To this end, *N*-hydroxy-2-thiopyridone esters are deemed suitable as radical affording substances by photo-induced rearrangement. The consequences of the interactions between the generated oxygen- or carbon-centered radicals and the matrices are expectedly prone to analytical observation by EPR of radical species generated in situ, and by NMR of adducts isolated by chromatography after radical reaction.

2. Materials and methods

2.1. Materials

Adamantyl carboxylic acid, pyridine-3-carboxylic acid, p-toluic acid, *N*-hydroxypyridine-2-thione, oxalyl chloride, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), $CDCl_3$, were purchased from Sigma–Aldrich Srl (Milan, Italy) and used without further purification. Solvents were of reagent grade. Aqueous solutions were made with demineralised water. DBC and MPC were prepared according to published protocols (Muzzarelli, Francescangeli, Tosi, & Muzzarelli, 2004; Muzzarelli et al., 2007) and shrimp chitosan with average MW 150 kDa as determined by laser light scattering, and degree of deacetylation 0.92 determined by alkalimetry was supplied by Primex, Siglufjordur, Iceland.

2.2. Methods

2.2.1. Preparation of *N*-acyloxy-pyridine-2-thione derivatives ***1a*** and ***1b***

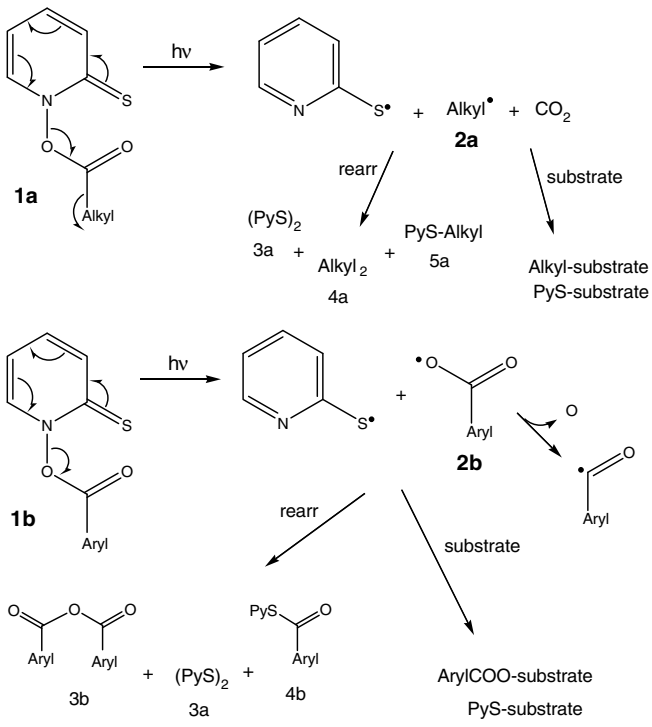
Compounds **1a–b** were prepared according to the procedure already described by Barton and co-workers (Barton, Crich, & Motherwell, 1985), concerning the reaction between equivalent amounts of the proper acyl chloride and 2-mercaptopyridine-*N*-oxide in anhydrous solvent. Further purification of the reaction mixture on silica gel through a short chromatographic column, afforded pure esters **1** as yellow solids. The structures of **1** were confirmed by H NMR and IR spectra.

2.2.2. Photochemical reactions and chromatography

As depicted in Scheme 1, PTOC esters undergo decarboxylation leading to the formation of alkyl radicals upon irradiation with visible light. As light source, we used a 300 W air flow-cooled tungsten lamp kept 15 cm away from the sample. A typical experimental procedure is given as follows: in a 100 ml round bottomed flask, 1.0 mmol of **1a** or **1b** were dissolved in 35 ml of anhydrous dichloromethane. The solution was kept protected against the light wrapping recipient by means of aluminum foil and 400 mg of DBC (or MPC or plain chitosan) were added at once. The atmosphere inside the flask was then saturated with nitrogen and the mixture allowed to stand for 1 h with occasional stirring. The solvent was then evaporated in vacuo at room temperature until a semi-solid residue was obtained and the residue exposed to the lamp light. Irradiation was carried out until thin layer chromatography showed that the esters **1** had completely disappeared (60 min). Raw mixtures were then submitted to chromatography on a 45 cm silica gel column, starting with hexane/ethyl acetate 3:1 and ending with ethyl acetate/methanol 95:5 as eluants.

2.2.3. Photolysis and EPR measurements

EPR spectra were recorded at 9.5 GHz on a Bruker EMX spectrometer equipped with a TE 102 cavity using a quartz aqueous flat cell. Unless otherwise indicated, the following instrumental settings were used: microwave power, 20 mW; modulation, 0.5 G; time constant, 0.25 s; scan time, 2 min. The samples were irradiated for 1 min directly inside the microwave cavity of the spectrometer using the above described apparatus. The spectra were processed on a PC by means of Bruker software and simulated using the program SIMFONIA by Bruker. The spectrum of an irradiated DMPO solution in the absence of any other component was recorded before the EPR analysis of each sample in the same experimental conditions as the sample. In the case that the spectrum of DMPO-alone sample showed a signal, this signal was subtracted from the DMPO + radical signal. Samples were dissolved in



Scheme 1. Photoinduced radical chain decarboxylation mechanism of acyl and aroyl derivatives of *N*-hydroxypyridine-2-thione (PTOC esters).

water/acetonitrile mixture in such a ratio that the best dissolution of materials has been accomplished.

The identification of the radical species was performed by comparing the hyperfine coupling constants (a_N and $a_{\beta H}$, accuracy from computation: ±0.03 G) with those reported in the literature. A very precious source of references is the spin-trap data base at NIEHS (http://mole.chm.bris.ac.uk/cgi-bin/stdb). The spin trap 5,5-dimethylpyrroline-*N*-oxide (DMPO) has emerged as well suitable for the identification of unstable, both carbon-centered and oxygen-centered intermediates, because the splitting from the β_H protons are sensitive to the nature of the trapped radical. This method has been extensively used for the detection and identification of short-lived free radicals in chemistry, biology, and medicine (Janzen & Blackburn, 1968; Motley & Mason, 1989; Rosen, Britigan, Halpern, & Pou, 1999).

2.2.4. ^{1}H NMR and IR spectra

Proton NMR spectra were measured at T = 290.0 K with a Bruker AC 200 instrument; $CDCl_3$ solutions were used for all experiments. All chemical shifts were relative to the TMS signal at 0.0 ppm. IR spectra were taken in Nujol on a Nicolet Avatar 360 FT-IR instrument.

3. Results and discussion

3.1. EPR experiments

Since the DMPO spin trap is well known to produce radicals by itself after irradiation, we first analyzed the spectra obtained, under the same experimental conditions, by irradiating the DMPO solution in the absence of the Barton's esters (PTOC esters) and the chitosan derivatives, or in the presence of each of these substances. We did not obtain any recordable EPR signal from these samples, with the exception of the **1b** (aryl = 3-pyridyl) solutions where a not negligible signal due to DMPO–OH· radicals appeared (not shown). This signal survived also in the presence of the different chitosans, and it was subtracted from the signals generated by solutions of **1b** and chitosans. The spectra thereafter shown in this paper are already "cleaned" from this signal.

The EPR spectra changed over time and the analysis as a function of the aging time allowed to identify a different kinetics for different radical species. Fig. 1 shows examples of the EPR spectra (normalized in intensity) of mixtures of DBC (saturated solution) and **1a** recorded at different delay times after irradiation: t = 0 min, t = 10 min; and t = 30 min. As discussed above, the solutions containing only DBC, in the presence of DMPO, did not show any EPR signal after irradiation. Conversely the mixture of DBC and ester **1a** showed an intense spectrum at t = 0 min. Interestingly, this spectrum was constituted by two components, whose relative intensities changed with time. At t = 30 min, only one component remained, which was subtracted from the spectrum at t = 0 min to obtain the other component (the subtraction was accomplished by considering a relative intensity of the two components of about 3:1). The prevailing component at t = 0 min was computed (dashed line, bottom of Fig. 1) with a_N = 15.5 G and $a_{\beta H}$ = 20.7 G. These parameters well correspond to those reported in the literature for a DMPO-carbon radical adduct (Julia, Bosch, Rodriguez, & Guerrero, 2000; Kadiiska, De Costa, Mason, & Mathews, 2000; Maples et al.,1988; Reszka & Chignell, 1995). In the present case the carbon radical was easily ascribed to the adamantyl radical.

The component which survived at t = 30 min was computed as shown on top of Fig. 1 (computation: dashed line) by means of the parameters: a_N = 13.6 G and $a_{\beta H}$ = 14.0 G. The literature reports equivalent parameters for DMPO-thiyl radicals, mainly for DMPO-phenylthiyl (Ito & Matsuda, 1984; Josephy, Rehorek, & Janzen, 1984). Therefore we attribute the signal at $t \geqslant 30$ min to the DMPO-PyS radical generated by photolysis with the mechanism reported in Scheme 1. The computation of the spectrum at t = 0 min (dotted line in Fig. 1) was therefore obtained by adding 30% of the DMPO-S radical signal and 70% of the DMPO-C radical signal.

These results indicate that: (a) the photolysis of ester **1a** produced radicals which were stabilized by interactions with the DBC: the increased life time was long enough to allow spin trapping of the radicals by DMPO; (b) the DMPO-carbon radical was prevalent, but its life time is shorter than that of the DMPO-SPy radical: after 30 min the DMPO-carbon radical had largely annihilated, whereas the DMPO-PyS radical was more persistent.

Similar results were found for MPC, but with some important differences. Fig. 2a shows the EPR spectra (superimposed and not

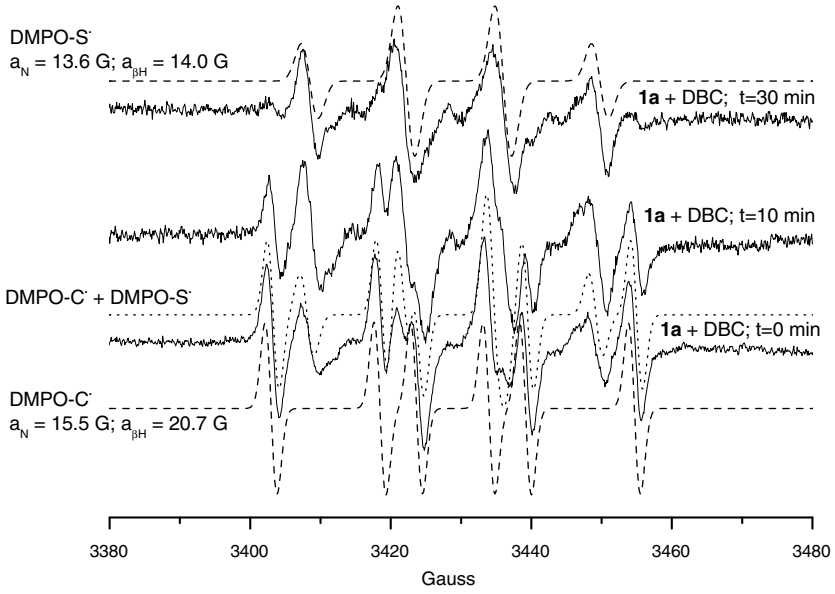


Fig. 1. EPR spectra of DBC (saturated solution) + adamantoyl-derivative **1a** (+DMPO), recorded at different delay times after irradiation: t = 0 min, t = 10 min; and t = 30 min. Bottom spectrum (dashed line): computation of the DMPO-C. signal; top spectrum (dashed line): computation of the DMPO-S. signal. Dotted line: computation of the spectrum at t = 0 min, obtained by adding 30% of the DMPO-S. signal and 70% of the DMPO-C. signal.

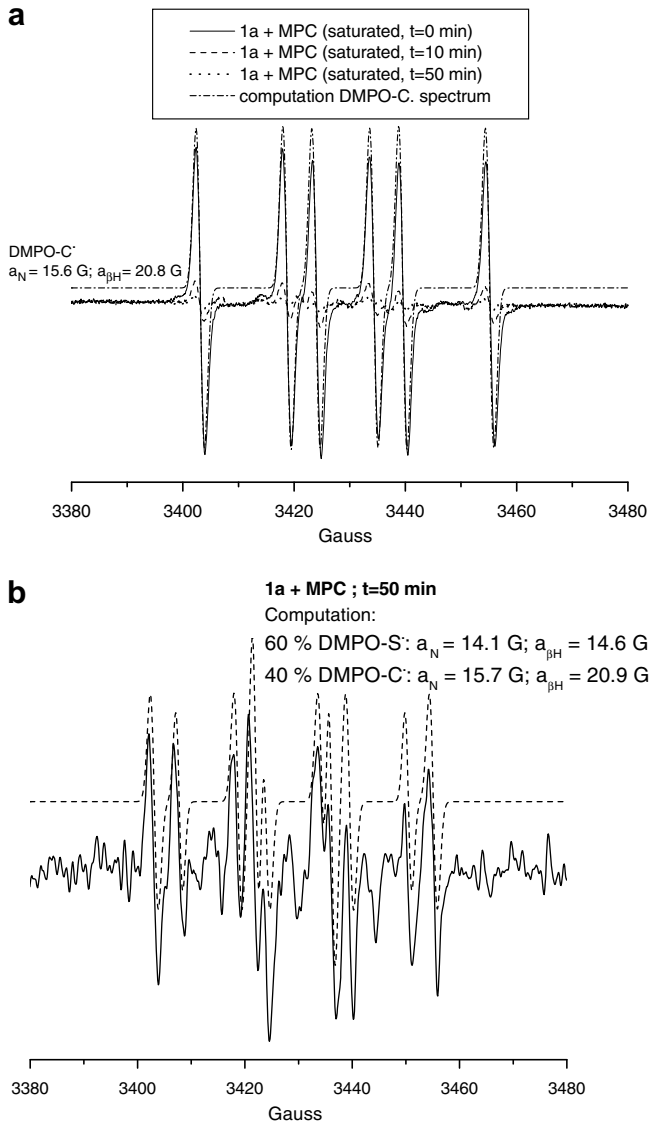


Fig. 2. (a) EPR spectra (superimposed to each other, and not normalized in intensity) of MPC (saturated solution) + adamantoyl-derivative **1a** (+DMPO), at t = 0 min (full line), t = 10 min (dashed line), and t = 50 min (dotted line). The dotted-dashed line shows the computation of the DMPO-C. signal (a_i parameters also reported in the figure). (b) EPR experimental (full line) and computed (dashed line) spectrum of MPC + adamantoyl-derivative **1a** (+DMPO) at t = 50 min. The relative percentages of the DMPO-C. and the DMPO-S. radicals and the a_i parameters used for computation are also reported in the figure.

normalized in intensity) of MPC (saturated solution) + **1a** at t = 0 min (full line), t = 10 min (dashed line), and t = 50 min (dotted line). The dotted-dashed line shows the computation of the DMPO-C radical signal by means of a_N = 15.6 G and $a_{\beta H}$ = 20.8 G. At t = 0 min, this DMPO-C radical signal was at high intensity (higher than that found for DBC), whereas the DMPO-PyS radical was at very low intensity. The DMPO-PyS radical signal became recognizable in the EPR spectra when the DMPO-C radical signal was largely annihilated, that is, in the spectrum recorded at t = 50 min, more clearly shown in Fig. 2b. This spectrum was computed (dashed line in Fig. 2b) considering 60% of DMPO-S radicals. It is interesting to note that the a_i parameters used for computation were slightly higher for MPC than for DBC and they increased with time. This increase reflects the increased environmental polarity of the nitroxide group (Ottaviani, Martini, & Nuti, 1987). Therefore, the radicals interacting with MPC (more polar) experienced a higher environmental polarity with respect to the radicals interacting with DBC (less polar).

To analyze more thoroughly the substrate effect on radical retention and scavenging, the intensities of the EPR signals were evaluated (by double integration of the peaks) and plotted *versus* time. Fig. 3a shows in the inset the plots of the DMPO-C radical intensity *versus* time for DBC and MPC. The data in these plots were obtained for saturated solutions diluted 1 to 1×10^{-4}, but similar plots were obtained at every concentration of the substrates: in all cases the intensity of the DMPO-C radical signal was higher for MPC than for DBC. Therefore the MPC structure trapped the DMPO-C radicals more effectively than DBC. This seems in contrast with the higher hydrophobicity of DBC, which is expected to better trap a hydrophobic radical. One should keep in mind however that DMPO is a polar molecule and, on the other side, a high local concentration of radicals onto the substrate favours the radical annihilation.

Fig. 3a also shows the DMPO-C radical intensity plots for three different concentrations of DBC, that is, the saturated solution, and the solutions diluted 1 to 1×10^{-4} (1E-4) and 1 to 1×10^{-8} (1E-8). Since the radicals are stabilized by interacting with the substrate,

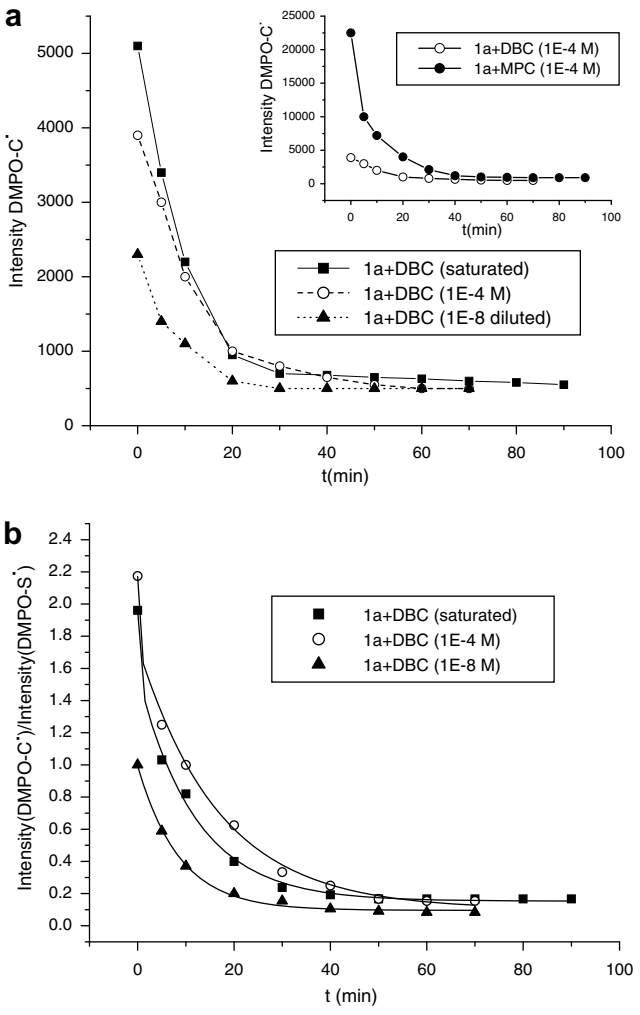


Fig. 3. (a) Variation of the intensity of the DMPO-C radical signal from **1a** as a function of the aging time after irradiation in the presence of DBC or MPC; filled squares: saturated solution of DBC; open circles: DBC, 1E-4 diluted; filled circles: MPC 1E-4 diluted; filled triangles: DBC, 1E-8 diluted. (b) Variation of the intensity ratio between the DMPO-C. and the DMPO-S. signals of **1a** as a function of the aging time after irradiation; filled squares: saturated solution of DBC; open circles: DBC, 1E-4 diluted; filled triangles: DBC, 1E-8 diluted.

the intensity of the DMPO-C radical signal diminished with the decrease of the substrate concentration, but not in a linear way: even highly diluted solutions still showed a relatively high intensity of the DMPO-C radical signal at $t = 0$ min. Furthermore, at $t = 30$ min, the intensity of the DMPO-C radical signal is higher for the 1E-4 dilution than for the undiluted solution. This suggests a sort of saturation of the interactions and a distribution of radicals in different interacting conditions, where the radicals experienced different life times. A further demonstration of this hypothesis is given by analyzing the variation of the intensity ratio between DMPO-C and DMPO-S radicals with time, reported in Fig. 3b. The 1E-4 dilution provokes a stabilization of the DMPO-C radicals with respect to the DMPO-S ones up to $t = 50$ min.

Different spectral components contributed to the EPR spectra when *N*-nicotinoyloxy-pyridine-2-thione **1b** (NPT), see Scheme 1 (aryl = 3-pyridyl), was irradiated in the presence of the different substrates and DMPO. Fig. 4 shows typical EPR spectra of DBC (Fig. 4a) and MPC (Fig. 4b) (saturated solution) + NPT and DMPO, at different delay times after irradiation (full line, $t = 0$ min; dashed line, $t = 10$ min; dotted line, $t = 50$ min). For both DBC and MPC, the $t = 0$ min spectrum showed a small contribution from a DMPO-C radical, computed with the a_i parameters indicated in the figure. The small but significant difference between these parameters and those calculated for the NPT systems indicate a different structure of this radical, which originated from the formation of a poorly stable acyl radical, as shown in Scheme 1. Therefore, this DMPO-C radical completely disappeared after few minutes.

Conversely, two other more persistent radical species contributed to the EPR signal at different relative intensities over time. The subtraction of the spectra at different delay times allowed to recover these two components. This is shown in Fig. 5a, where the experimental signals (full lines) are superimposed to the computed signals (dashed lines: the a_i parameters are also shown in the figure) for DBC and MPC saturated solutions + NPT and DMPO. These two components were attributed to DMPO-carboxyl and DMPO-peroxyl radicals on the basis of the a_N and $a_{\beta H}$ obtained from the computation of the spectra and compared to the literature parameters (Dabestani, Hall, Sik, & Chignell, 1990; Harbour

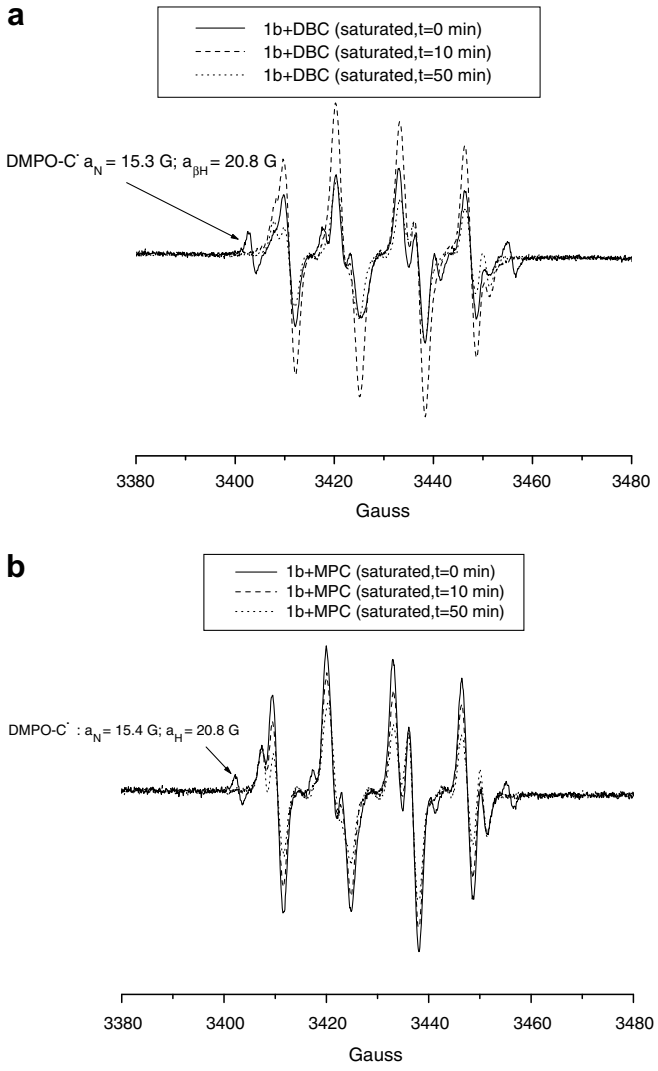


Fig. 4. (a) EPR spectra of DBC (saturated solution) + nicotinoyl-derivative (NPT) **1b** (+DMPO): full line, $t = 0$ min; dashed line, $t = 10$ min; dotted line, $t = 50$ min. (b) EPR spectra of MPC (saturated solution) + NPT **1b** (+DMPO): full line, $t = 0$ min; dashed line, $t = 10$ min; dotted line, $t = 50$ min.

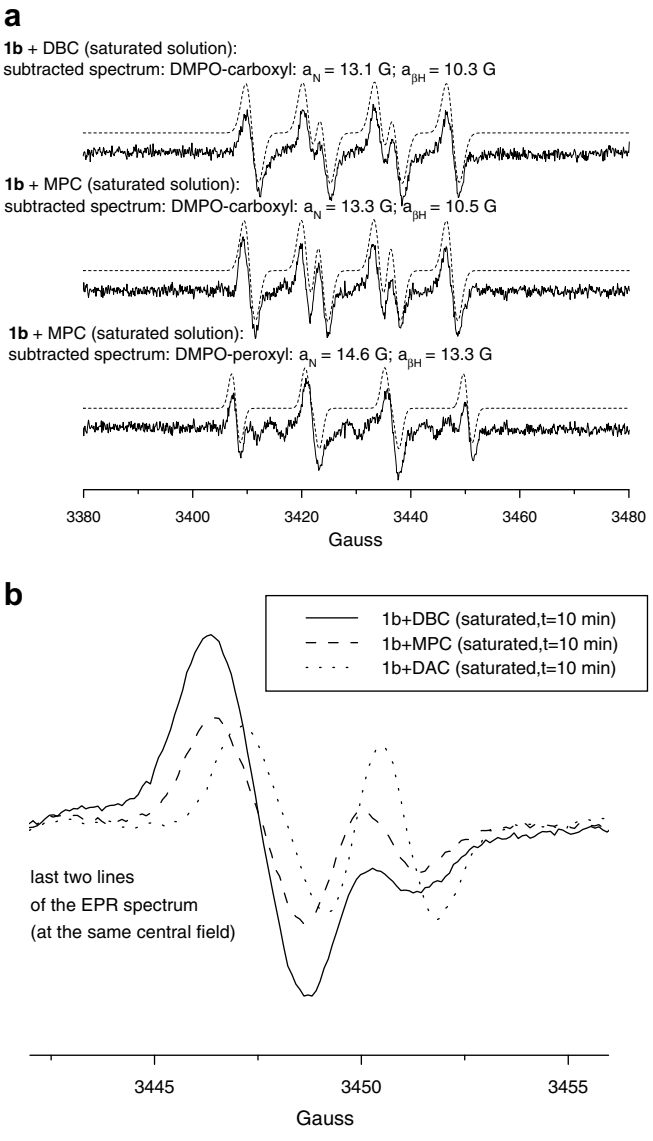


Fig. 5. (a) Main EPR spectra components obtained from subtraction of spectra at different delay times after irradiation of DBC and MPC saturated solutions + **1b** and DMPO; full lines: experimental subtracted components; dashed lines: computed signals (the a_i parameters used for computation are also reported). (b) High-field portion of the spectra recorded for **1b** in the presence of the three different substrates MPC, DBC and deacylated-chitosan (DAC) (saturated solutions), at $t = 10$ min.

& Hair, 1978; Kalyanaraman, Mottley, & Mason, 1984; Reszka & Chignell, 1983; Schaich & Borg, 1988). The signal of DMPO-peroxyl radical for the DBC system is not reported for sake of clarity, but the same increase of the a_i parameters from MPC to DBC was found for both carboxyl and peroxyl radicals. This increase is in line with the increased environmental polarity of the radicals present at the substrate surface of MPC, with respect to DBC. In this respect, we wanted to perform a comparison also with a more polar chitosan that is a 92% deacetylated chitosan, in the presence of NPT and DMPO. Fig. 5b shows the two lines at the highest field of the EPR spectra of NPT in the presence of DBC (full line), MPC (dashed line) and deacylated-chitosan (DAC) (dotted line) (saturated solutions) at t = 10 min. These lines were progressively shifted to higher fields because of the increase of the a_i values, which in turn follows the increase of the substrate polarity.

Fig. 6a shows the variation of the absolute intensity of the DMPO-carboxyl signal as a function of time, whereas Fig. 6b shows the variation of the intensity ratio between the DMPO-carboxyl and the DMPO-peroxyl radicals over the aging time, in the presence of DBC and MPC. We expected the polar DMPO-carboxyl radical be stabilized at the more polar chitosan substrate, but we found the opposite: the intensity of the DMPO-carboxyl signal, evaluated both as absolute intensity (Fig. 6a) and as relative intensity (Fig. 6b), was higher for DBC with respect to MPC. This holds in all the range of substrate concentrations (in Fig. 6a the values are at 1E-8 dilution; whereas in Fig. 6b the relative intensity values are at 1 E-4 dilution). The difference among the various dilutions of the substrate mainly resides in a higher concentration of the DMPO-carboxyl radicals for the undiluted samples with respect to the diluted ones (Fig. 6c). Therefore, even at high delay times, such as t = 100 min, the intensity of the DMPO-carboxyl radical at the DBC surface is still high, indicating the stabilization of these radicals in the chitin substrate. We hypothesize that the DBC substrate hosts the radicals in protected and poorly reactive environment, whereas hydrophilic chitosan substrates favour a more rapid annihilating of the radicals as a consequence of collisions with their radicophilic sites (e.g. free OH and NH_2 groups). This hypothesis has been confirmed by results of chemical reactivity experiments.

3.2. Chemical reactivity experiments

N-Hydroxy-2-thiopyridone esters of aliphatic and aromatic acids (Barton's PTOC esters) **1a**, **1b** (Scheme 1) are a convenient source of carbon- or oxygen-centered radicals, respectively. When irradiated with a tungsten lamp or sunlight, they quickly undergo a one-electron rearrangement with generation of alkyl or aroyloxy radicals according to the mechanism depicted in Scheme 1 (Barton et al., 1985; Barton & Ramesh, 1990).

PTOC esters have been widely employed as non-conventional synthetic methodology for the preparation of organic compounds (Barton & Parekh, 1993). Unlike hydroxyl radicals or carbon-centered radicals generated by conventional hydrophilic sources (hydrogen peroxide, alkyl hydro-peroxides, diazo-compounds), PTOC esters give rise to radical species, such as **2a** or **2b**, whose structure can be chemically recognized in substrates with which they were made to react. In the presence of radicophilic substrates they work as chemical markers of those sites with which they are prone to interact. The adducts, when isolated and characterized, permit to achieve otherwise hardly obtainable information about the substrate reactivity and the reaction mechanism.

However, in the absence of radicophilic substrates, photolysis of PTOC esters usually leads to the coupling of firstly originated radicals with the formation of the rearrangement products **3a**, **4a**, **5a** from **1a** and **3a**, **3b**, **4b** from **1b** (see Scheme 1) (Barton et al., 1985; Barton & Ramesh, 1990). These compounds are easily detected by NMR and IR spectroscopy.

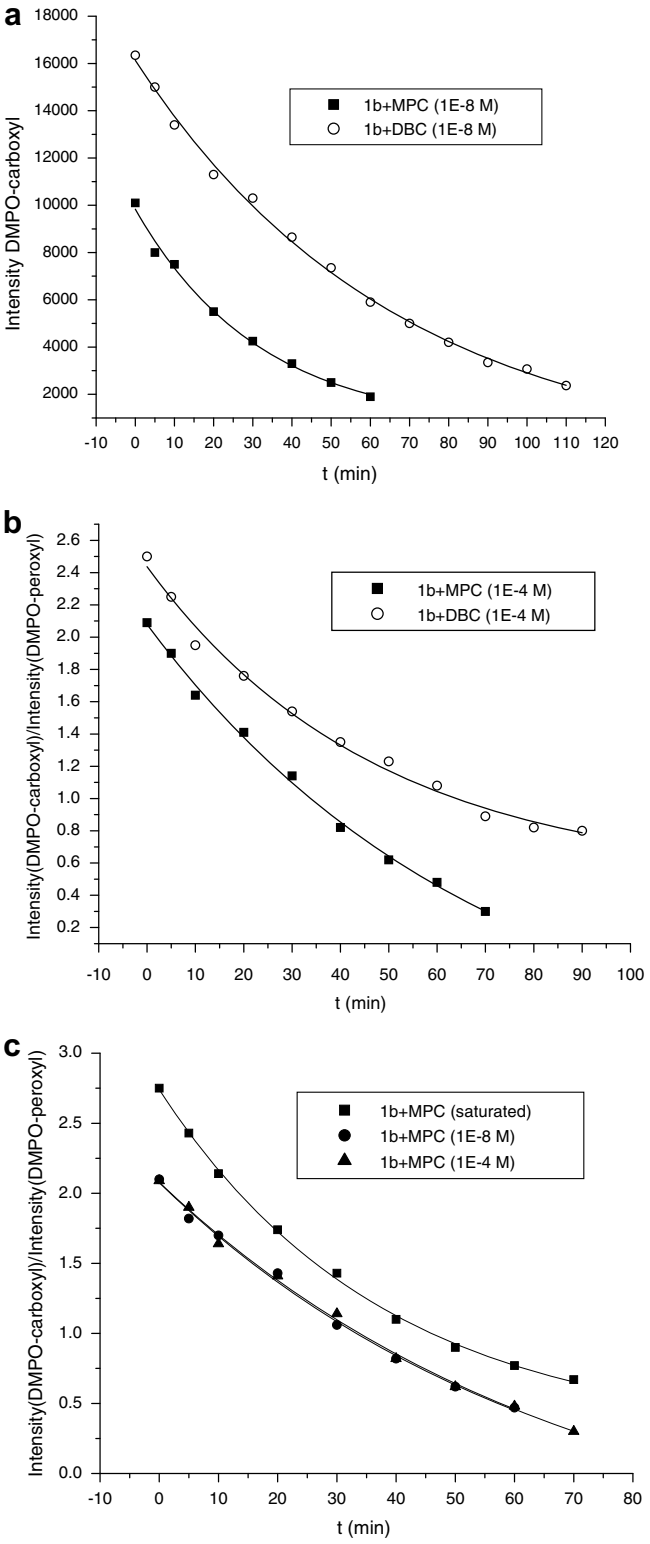


Fig. 6. (a) Variation of the absolute intensity of the DMPO-carboxyl signal as a function of time for MPT **1b** + DBC (empty circles) and MPC (filled squares) (1E-8 diluted). (b) Variation of the intensity ratio between the DMPO-carboxyl and the DMPO-peroxyl radicals over the aging time, for MPT **1b** + DBC (empty circles) and MPC (filled squares) (1E-4 diluted). (c) Variation of the intensity ratio between the DMPO-carboxyl and the DMPO-peroxyl radicals over the aging time, for MPT **1b** - + MPC at three concentrations (undiluted: filled squares; 1E-4: filled triangles; 1E-8 diluted: filled circles).

A recently accomplished study on PTOC as RAS (radical affording substance) in aqueous media (Castagnino, Cangiotti, Tongiani,

& Ottaviani, 2005), showed that **1a** and congeners release, by photolysis, radicals which can find efficient stabilization in presence of polysaccharide matrices. The fate of such radicals depends on the reactivity of substrate (its radicophilicity) on one hand but also on the electronic features of radical species themselves: **2a** is a nucleophilic-in-character radical whereas **2b** is an electrophilic intermediate. This means that **2a** and **2b** found the most proper way to react, and consequently manifested their shortest life time upon interaction with an electronic complementary-in-character substrate, electrophilic and nucleophilic, respectively. Free –OH and $–NH_2$ chitosan groups are source of hydrogen radicals, so that ungrafted and hydrosoluble chitosans could more promptly interact with electrophilic radicals, oxygen-centered radicals actually, than towards nucleophilic ones.

The above reported results of EPR experiments carried out on MPC irradiated in the presence of **1a** (alkyl = adamantyl) or **1b** (aryl = 3-pyridyl) are in good agreement with such rationale. Figs. 3a and 6a show that methylpyrrolidinone chitosan (MPC) exhibited radical scavenging activity towards both nucleophilic and electrophilic radicals respectively. However, MPC, being a nucleophilic matrix prone to afford hydrogen radicals from free –OH and $–NH_2$ groups, clearly reacts with electrophilic radicals (from **1b**) faster then towards the nucleophilic ones (from **1a**), the latter being still present inside the matrix 90 min after irradiation (Fig. 3a). We expected that DBC, bereft of free alcoholic and aminic hydrogen atoms, should exhibit negligible activity when submitted to the same experimental conditions. On the contrary, DBC showed a remarkable radical scavenging activity of the same magnitude of that exhibited by MPC (Figs. 1 and 3a).

In the attempt to rationalize the activity pointed out through the EPR experiments, DBC and MPC were irradiated in the presence of **1a** (alkyl = adamantyl) or **1b** (aryl = 3-pyridyl, p-tolyl) and raw mixtures submitted to purification by flash chromatography.

Since *N*-nicotinoyloxy-pyridine-2-thione **1b** (NPT) and its rearrangements by-products, thioester **4b** and disulphide **3a** give, when in mixture, crowded H NMR spectra in the region 8.4–7.2 δ with broad overlapping of signals, we preferred to carry out experiments in the presence of the p-tolyl congener of **1b**, whose reaction products gave rise to easier attributable spectra. Unless otherwise specified, we refer to *N*-p-tolylcarboxy-pyridine-2-thione when compound **1b** is cited in this section. Solid mixtures of chitins and esters were prepared as described in the Section 2.2 and then submitted to irradiation under inert atmosphere until the esters completely reacted. Without further work up, raw mixtures were then submitted to a silica gel chromatography by using hexane/ethyl acetate 3:1 as starting eluant. When **1a** was used as reagent on DBC, adamantyl-pyridyl-sulphide **5a** was isolated as the major product (0.82 mmol, H NMR, δ: 8.5, m, 1H; 7.5, m, 1H; 7.4, m, 1H; 7.1, m, 1H; 2.1, m, 9H; 1.7, m, 6H), whereas disulphide **3a** was collected in negligible amount. It is noteworthy that adamantyl-pyridyl-sulphide **5a** that otherwise should form in poor amount together with **3a** and **4a**, in the presence of DBC formed in almost quantitative yield with respect to PTOC **1a**. Elution with ethyl acetate/methanol 95:5 allowed unreacted DBC to be recovered with traces of adducts due to the reaction of **1a** with substrate. On the other hand, when **1b** was irradiated in the presence of DBC, no trace of such addition compounds was detected, but anhydride **3b** (H NMR, δ: 8.05, d; 7.40, d; 2.49, s; IR: 1774, 1711, 1038 cm^{-1}) and dipyridyl-disulphide **3a** (Barton et al., 1985; Barton & Ramesh, 1990) were collected as sole products.

Despite of its scarce reactivity towards **1a**, quite different results were obtained when MPC was photolysed in the presence of *N*-p-tolyl-carboxypyridine-thione **1b**. In this case the less polar chromatographic fractions contained p-toluic-anhydride **3b**, thioester **4b** (H NMR, δ: 8.51, d; 8.0, d; 7.65, m; 7.28, d; 7.16, m; 2.44, s) and disulphide **3a**, whereas the fractions eluted with ethyl acetate/methanol afforded a complex mixture of products whose NMR analysis allowed to ascertain the presence of carbo- or carboxy-p-tolyl groups linked to some glucosyl-like framework compounds (H NMR, δ: 7.95, d; 7.70, d; 7.35, d; 7.24, d; 6.35, m; 4.11, m; 3.46, m; 2.40, s; 2.35, s; 1.9–0.9, large m; IR: 1705, 1690, 1675 cm^{-1}). This fact clearly shows that, unlikely DBC, MPC structurally participates to the chemical interactions among radical species probably involving hydrogen atoms on free –OH and $–NH_2$ groups, according to the mechanism proposed by Park et al. (2004). An analogue photochemical experiment carried out on chitosan in the presence of **1b** confirmed the behaviour observed for MPC. In the case of chitosan, comparative TLC analysis of the two reaction mixtures showed the formation of the same radical rearrangement products **3a**, **3b** and **4b** together with the presence of the mixture of more polar compounds in rather similar composition.

On the basis of chemical behaviour we are able to report that hydrophilic chitosans, i.e. chitosans having unsubstituted –OH and $–NH_2$ groups, possess good radical scavenging activity towards electrophilic radicals like **2b** mainly with a mechanism that chemically involves their structure in intra-molecular radical rearrangements. On the opposite, the highly esterified lipophilic dibutyryl chitin DBC, has shown to be endowed with the same scavenging activity without being involved into framework fragmentation and propagation of the radical cascade.

4. Conclusions

Chitins and chitosans were found to be able to annihilate radicals with a mechanism that not always sees them chemically involved with structural modifications and with radical cascade propagation. The application of reactivity of PTOC esters described in this work as radical affording substances onto chitopolysaccharides, allows to point out that radical scavenging properties of such polymers not only depend on the presence of free alcohol and amino groups as hydrogen atom donors but also on the ability of these matrices to work as radical cages, entrapping and constraining free radicals to undergo copulation reactions. The copulation of radicals is the most effective process leading to the quenching of a radical chain reaction but it is a rare event when radicals are generated *in vivo* where they can freely interact with biochemical environment affording irreversible damages. There is interest in protecting delicate biochemical environments against radicals with the aid of modified chitosans (Ji et al., 2007; Mendis, Kim, Rajapakse, & Kim, 2007; Rajapakse, Kim, Mendis, & Kim, 2007).

This study demonstrates that modified polysaccharides, MPC and DBC in our case, behave as effective radical scavengers since they are able to prevent the propagation of chain reaction onto polymeric framework when free radical species are generated inside the matrix. Such behaviour, that can be related to the electronic character of the radical species (nucleophilic or electrophilic) and to the degree of substitution of the polysaccharides, becomes furthermore interesting when one considers that a couple of radicals usually arises when a chemical bond is cleaved as a consequence of photolytic events. In this case, the presence of modified polysaccharides such as DBC, would force radicals to copulate on themselves, preventing the damage induced by their propagation. It is deemed that the present information is useful for further developing functional biomaterials (Muzzarelli et al., 2007).

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References

Barton, D. H. R., Crich, D., & Motherwell, W. B. (1985). The invention of new radical chain reactions. Part VIII. Radical chemistry of thiohydroxamic esters; a new method for the generation of carbon radicals from carboxylic acids. *Tetrahedron, 41*, 3901–3908.

Barton, D. H. R., & Parekh, S. I. (1993). *Half a century of free radical chemistry*. Cambridge: University Press [pp. 46–146].

Barton, D. H. R., & Ramesh, M. (1990). Generation and fate of nondecarboxylating acyloxy radicals derived from the photolysis of acyl derivatives of *N*-hydroxy-2-thiopyridone. *Tetrahedron Letters, 31*, 949–952.

Castagnino, E., Cangiotti, M., Tongiani, S., & Ottaviani, M. F. (2005). A study of free radical release from β-cyclodextrin-anticancer pro-drugs adducts in water. *Journal of Controlled Release, 108*, 215–225.

Chen, Q., Liu, S. Q., Du, Y. M., Peng, H., & Sun, L. P. (2006). Carboxymethyl-chitosan protects rabbit chondrocytes from interleukin-1beta-induced apoptosis. *European Journal of Pharmacology, 541*, 1–8.

Dabestani, R., Hall, R. D., Sik, R. H., & Chignell, R. H. (1990). Spectroscopic studies of cutaneous photosensitizing agents-XV. Anthralin and dihydroxyanthraquinone. *Photochemistry and Photobiology, 52*, 961–971.

Esumi, K., Takei, N., & Yoshimura, T. (2003). Antioxidant potentiality of gold–chitosan nanocomposites. *Colloids and Surfaces B: Biointerfaces, 32*, 117–123.

Guo, Z., Liu, H., Chen, X., Ji, X., & Li, P. (2007). Hydroxyl radical scavenging activity of *N*-substituted chitosan and quaternized chitosan. *Bioorganic & Medicinal Chemistry Letters, 16*, 6348–6350.

Guo, Z., Xing, R., Liu, S., Yu, H., Wang, P., Li, C., & Li, P. (2005). The synthesis and antioxidant activity of the Schiff bases of chitosan and carboxymethyl chitosan. *Bioorganic & Medicinal Chemistry Letters, 15*, 4600–4603.

Harbour, J. R., & Hair, M. L. (1978). Detection of superoxide ions in nonaqueous media. Generation by photolysis of pigment dispersions. *Journal of Physical Chemistry, 2*, 1397. Available from http://mole.chm.bris.ac.uk/cgi-bin/stdb.

Huang, R., Rajapakse, N., & Kim, S. K. (2006). Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives. *Carbohydrate Polymers, 63*, 122–129.

Ito, O., & Matsuda, M. (1984). Flash photolysis study for substituent and solvent effects on spin-trapping rates of phenythiyl radicals with nitrones. *Bulletin of the Chemical Society of Japan, 57*, 1745–1749.

Janzen, E. G., & Blackburn, B. J. (1968). Detection and identification of short-lived free radicals by an electron spin resonance trapping technique. *Journal of the American Chemical Society, 90*, 5909–5910.

Je, J. Y., Cho, Y. S., & Kim, S. K. (2006b). Cytotoxic activity of water-soluble chitosan derivatives with different degree of deacetylation. *Bioorganic & Medicinal Chemistry Letters, 16*, 2122–2126.

Je, J. Y., & Kim, S. K. (2006a). Antioxidant activity of novel chitin derivative. *Bioorganic & Medicinal Chemistry Letters, 14*, 5989–5994.

Ji, X., Zhong, Z. M., Chen, X. L., Xing, R. G., Liu, S., Wang, L., & Li, P. C. (2007). Preparation of 1,3,5-thiadiazine-2-thione derivatives of chitosan and their potential antioxidant activity in vitro. *Bioorganic & Medicinal Chemistry Letters, 17*, 4275–4279.

Josephy, P. D., Rehorek, D., & Janzen, E. G. (1984). Electron spin resonance spin trapping of thiyl radicals from the decomposition of thionitrites. *Tetrahedron Letters, 25*, 1685–1688.

Julia, L., Bosch, M. P., Rodriguez, S., & Guerrero, A. (2000). Direct evidence of a radical mechanism in the addition reaction of iododifluoroesters to olefins by spin trapping. *Journal Organic Chemistry, 65*, 5098–5103.

Kadiiska, M. B., De Costa, K. S., Mason, R. P., & Mathews, J. M. (2000). Reduction of 1,3-diphenyl-1-triazene by rat hepatic microsomes, by cecal microflora and in rats generates the phenyl radical metabolite. An ESR spin trapping investigation. *Chemical Research and Toxicology, 13*, 1082–1086.

Kalyanaraman, B., Mottley, C., & Mason, R. P. (1984). On the use of organic extraction in the spin-trapping technique as applied to biological systems. *Journal of Biochemical and Biophysical Methods, 9*, 27–31.

Lin, H. Y., & Chou, C. C. (2004). Antioxidative activity of water-soluble disaccharides chitosan derivative. *Food Research International, 37*, 883–889.

Maples, K. R., Jordan, S. J., & Mason, R. P. (1988). In vivo haemoglobin thiyl free radical formation following administration of phenylhydrazine and hydrazine-based drugs. *Drug Metabolism and Disposition, 16*, 799–803.

Matsugo, S., Mizuie, M., Matsugo, M., Ohwa, R., Kitano, H., & Konishi, T. (1998). Synthesis and antioxidant activity of water-soluble chitosan derivatives. *IUBMB Life, 44*, 939–948.

Mendis, E., Kim, M. M., Rajapakse, N., & Kim, S. K. (2007). An in vitro cellular analysis of the radical scavenging efficacy of chitooligosaccharides. *Life Sciences, 80*, 2118–2127.

Motley, C., & Mason, R. P. (1989). *Biological Magnetic Resonance*. New York: Plenum [pp. 489–546].

Muzzarelli, C., Francescangeli, O., Tosi, G., & Muzzarelli, R. A. A. (2004). Susceptibility of dibutyryl chitin and regenerated chitin fibres to deacylation and depolymerization by lipases. *Carbohydrate Polymers, 56*, 137–145.

Muzzarelli, R. A. A., Guerrieri, M., Goteri, G., Muzzarelli, C., Armeni, T., Ghiselli, R., et al. (2005). The biocompatibility of dibutyrylchitin in the context of wound dressing. *Biomaterials, 26*, 5844–5854.

Muzzarelli, R. A. A., Morganti, P., Morganti, G., Palombo, P., Palombo, M., Biagini, G., et al. (2007). Chitin nanofibrils/chitosan glycolate composites as wound medicaments. *Carbohydrate Polymers, 70*, 274–284.

Ottaviani, M. F., Martini, G., & Nuti, L. (1987). Nitrogen hyperfine splitting constant of nitroxide solutions: differently structured and charged nitroxides as probes of environmental properties. *Magnetic Resonance Chemistry, 25*, 897–904.

Park, P. J., Je, J. Y., & Kim, S. K. (2004). Free radical scavenging activities of differently deacetylated chitosan using ESR spectrometer. *Carbohydrate Polymers, 55*, 17–22.

Rajapakse, N., Kim, M. M., Mendis, E., & Kim, S. K. (2007). Inhibition of free radical-mediated oxidation of cellular biomolecules by carboxylated chitooligosaccharides. *Bioorganic & Medicinal Chemistry Letters, 15*, 997–1003.

Reszka, K., & Chignell, C. F. (1983). Spectroscopic studies of cutaneous photosensitizing agents-IV. The photolysis of benoxaprofen and anti-inflammatory drugs with phototoxic properties. *Photochemistry and Photobiology, 38*, 281.

Reszka, K. J., & Chignell, C. F. (1995). Photochemistry of 2-mercaptopyridines. EPR study of photoproduction of hydroxyl radicals by *N*-hydroxypyridine-2-thione using DMPO in aqueous solutions. *Photochemistry and Photobiology, 61*, 269–275.

Rosen, G. M., Britigan, B. E., Halpern, H. J., & Pou, S. (1999). *Free radicals: biology and detection by spin trapping*. Oxford: University Press.

Schaich, K. M., & Borg, D. C. (1988). Fenton reactions in lipid phases. *Lipids, 26*, 570–579.

Sun, T., Xu, P., Liu, Q., Xue, J., & Xie, W. (2003). Graft copolymerization of methacrylic acid onto carboxymethyl chitosan. *European Polymer Journal, 39*, 189–192.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters, 11*, 1699–1701.

Xing, R., Liu, S., Yu, H., Zhang, Q., Li, Z., & Li, P. (2004). Preparation of low-molecular-weight and high-sulfate-content chitosans under microwave radiation and their potential antioxidant activity in vitro. *Carbohydrate Research, 339*, 2515–2525.