Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Polymers 53 (2003) 109–115
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Selective reactivity of biochemically relevant quinones towards chitosans

Riccardo A.A. Muzzarelli[a,*], Giampaolo Littarru[a], Corrado Muzzarelli[a], Giorgio Tosi[b]

[a]*Institute of Biochemistry, Faculty of Medicine, University of Ancona, Via Ranieri 67, IT-60100 Ancona, Italy*
[b]*Department of Materials Science, Faculty of Engineering, University of Ancona, Via Brecce Bianche, IT-60100 Ancona, Italy*

Received 19 August 2002; revised 10 December 2002; accepted 28 December 2002

Abstract

Biologically significant quinones, such as menadione (vitamin K), plumbagin, ubiquinone (CoQ_{10}) and CoQ_3 were examined along with 1,2-naphthoquinone and 1,4-naphthoquinone for their capacity to react with five chitosans in freeze-dried or film form. The chitosans tested were: chitosan acetate salt, re-acetylated chitosan, amorphous chitosan, 5-methylpyrrolidinone chitosan and N-carboxymethyl chitosan. It was surprisingly found that CoQ_{10} and CoQ_3 do not react with the chitosans, whilst menadione and even more 1,4-naphthoquinone are reactive and yield deeply coloured modified chitosans. The reactivities of plumbagin and 1,2-naphthoquinone are modest or nil, depending on the chitosan tested. The maximum capacity of chitosans for 1,4-naphthoquinone corresponded to an amine/quinone molar ratio close to 2, indicative of saturation over a 12 h contact period: the relevant infrared spectra did not show the typical bands of 1,4-naphthoquinone. Uv–vis measurements on methanol solutions indicated that chitosan acetate salt and re-acetylated chitosan were most reactive with menadione. Menadione-treated chitosans gave infrared spectra containing typical quinone bands, and the films had altered surface properties, their contact angles with saline being much higher than for controls. The absence of reactivity between ubiquinone and *N*-carboxymethyl chitosan, both widely accepted functional cosmetic ingredients, could constitute the basis for the formulation of toot-pastes and gingival gels, possessing enhanced reparative properties due to the synergistic actions of intact ubiquinone and *N*-carboxymethyl chitosan.
© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosans; *N*-Carboxymethyl chitosan; Ubiquinone; Coenzyme Q_{10}; Plumbagin; Naphthoquinone; Menadione

1. Introduction

Quinones are reactive towards various nucleophiles including amines. In nature, certain quinones are generated enzymatically, and immediately react with available amines. For example, the adhesion of marine animals to submerged surfaces involves a protein with high dihydroxyphenylalanine (DOPA) content: the DOPA units are oxidised by tyrosinase and undergo cross-linking reaction with amino groups on the protein. The extremely rapid cross-linkage confers cohesive strength to the mussel glue (Sugumaran, 1998; Holl, Schaefer, Goldberg, Kramer, Morgan, & Hopkins, 1992).

Tyrosinases, laccases and phenol oxidases have therefore been used to generate nascent quinones in situ from DOPA and from accessible tyrosyl grafted phenolic units (Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994). For practical applications, it is of interest to react quinones with chitosan in the presence of tyrosinase and atmospheric oxygen: immediate perception of the reaction can be obtained by observing the browning of a chitosan film or the viscosity increase of a chitosan salt solution leading to gel formation (Chen, Kumar, Harris, Smith, & Payne, 2000; Kumar, Bristow, Smith, & Payne, 2000) or alteration of the chitosan solubility (Kumar, Smith, & Payne, 1999), besides instrumental evidence provided by IR, NMR, contact angle measurements, and dissolved oxygen measurements.

Several models have been studied for the use of chitosan for the removal of phenols from industrial waters in the presence of one of the mentioned enzymes (Payne, Sun, & Sohrabi, 1992; Sun, Payne, Maes, Chu, & Wallace, 1992): for example, removal of phenolic polymerization inhibitors (Patel, Sun, & Payne, 1994) and removal of phenolics from fermentation streams (Payne & Sun, 1994; Edwards, Leukes, Rose, & Burton, 1999).

The phenols taken into consideration in the cited studies were volatile phenols such as p-cresol (Wu, Chen, Wallace,

* Corresponding author. Tel.: +39-071-2204684; fax: +39-071-2204683.
E-mail address: muzzarelli@unian.it (R.A.A. Muzzarelli).

0144-8617/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0144-8617(03)00006-7

Vazquez-Duhalt, & Payne, 2001), gallate esters such as dodecyl gallate (Vachoud, Chen, Payne, & Vazquez-Duhalt, 2001), phenol (Sun and Payne, 1996), hexyloxyphenol (Chen et al., 2000), chlorogenic acid (Kumar et al., 1999), 1,2-benzenediol (Sun et al., 1992), *tert*-butylcathecol (Patel et al., 1994), *p*-hydroxyphenoxyacetate (Payne & Sun, 1994), olive-mill wastewater phenolics (Vachoud et al., 2001), and polyhydroxystyrene (Shao et al., 1999).

From this list of phenols/quinones, it appears that attention has been directed to chemically important models so far, but biochemically significant quinones were not studied in conjunction with chitosan.

Vitamin K quinones actually are compounds of major importance from the biochemical standpoint, being involved in modification of blood coagulation factors. Menadione, 2-methyl-1,4-naphthalenedione is a synthetic naphthoquinone derivative having the physiologic properties of vitamin K, a collective name including phylloquinones K_1, menaquinones K_2 and menadione K_3. Phylloquinone, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-1,4-naphthoquinone, actually is a photosynthetic electron carrier synthesized in green plants, a major dietary source of protrombogenic vitamin K.

Ubiquinone, also called coenzyme Q (CoQ_{10}), is a lipid soluble benzoquinone for which a definite biological role was first recognised in the mitochondrial respiratory chain (Mitchell, 1976). It is a 2,3-dimethoxy-5-methyl benzoquinone with a variable length terpenoid side chain containing from one to twelve mono-unsaturated *trans*-isoprenoid units. In mammals, nine and ten isoprenoid units are most common; CoQ_{10} is the ubiquinone present in human tissues. In the years following its discovery it was also found in cellular membranes other than mitochondria, such as in Golgi apparatus and plasma membrane. Its antioxidant properties are mainly ascribed to the reduced form of CoQ_{10}, i.e. ubiquinol (Ernster & Beyer, 1991). Several enzymes capable of reducing ubiquinone to ubiquinol are known (Kishi, Takahashi, Usui, Hashizume, & Okamoto, 1999). Recently, the antioxidant role of CoQ_{10} was very deeply inquired in plasma lipoproteins; in fact ubiquinol 10 is recognised as the most reactive lipophilic antioxidant in low density lipoproteins (Stocker, Bowry, & Frei, 1991). Its antioxidant properties are also exerted in the skin, which is notoriously exposed to high levels of oxidative stress (Hoppe et al., 1999; Passi, De Pita, Puddu, & Littarru, 2002). CoQ_{10} is administered to humans orally or, in the case of skin, by topical application.

Other compounds of potential interest are plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone, an antimicrobial very toxic agent from *Plumbago indica*; diclone, 2,3-dichloro-1,4-naphthoquinone, used in the past to protect seeds and depress algal growth in swimming pools; phthiocol, 2-hydroxy-3-methl-1,4-naphthoquinone, an antibiotic substance produced by *Mycobacterium tuberculosis*; and bupravacone, an antibiotic used in clinical experiments against *Cryptosporidium parum*. Menaquinones are a group of prenylated quinones.

In the present context, it is worth to underline the reparative capacities of chitosan and certain modified chitosans when applied to wounded animal tissues. Their activities on fibroblasts (Okamoto et al., 2002), macrophages (Muzzarelli, 1997) and neutrophils (Hua, Sakamoto, & Nagaoka, 2002), and rebuilding of glycosaminoglycans explain the good results in the wound healing with methyl pyrrolidinone chitosan even in difficult cases such as leg ulcers in aged patients (Mancini, Muzzarelli, & Mancini, 2000) and bone regeneration in large bone defects (Muzzarelli et al., 1993). Chitosans have a well documented hemostatic action (Klokkevold, Fukayama, Sung, & Bertolami, 1999; Kulling et al., 1999) due to their capacity to aggregate cells and to form polyelectrolyte complexes with mucins (Harding, 1997). *N*-Carboxymethyl chitosan is particularly suited for the preparation of cosmeceuticals and is being used since a few years for the formulation of hydrating creams.

The aim of the present work is to define the reactivity of some quinones of biological interest towards a selection of chitosans currently used in the cosmeceutical field.

2. Experimental

2.1. Chitosans and chemicals

The following chitosans were prepared from food grade crustacean chitosan supplied by Giusto Faravelli, Milano, Italy (degree of deacetylation 0.97; viscosity of 1% solution 110 mPa s; ashes 0.3%): *Chitosan acetate salt*, obtained from chitosan flakes (20 g) dissolved in acetic acid (20 g) in water (980 g), and dialysed against water in a dialysis tube with cut-off 1500. *Precipitated chitosan* obtained from chitosan flakes (120 g) suspended in water (10 l) dissolved with acetic acid (90 g) and treated with NaOH (75 g in 2.3 l) to precipitate chitosan at pH 8.05. The gel was filtered on a Buchner funnel through filter paper and washed with water. *Reacetylated chitosan*, obtained from chitosan (5 g) suspended in water (450 g), dissolved with acetic acid (5 g), treated with acetic anhydride (2.86 ml) in methanol (214 g) for 24 h under stirring, dialyzed and freeze-dried. The analytical data were obtained as previously described by Dal Pozzo et al. (2000). The 5-*Methyl pyrrolidinone chitosan* (MP-chitosan) was obtained from chitosan flakes (27 g) suspended in water (990 g), dissolved with levulinic acid (29.3 g), reduced with $NaBH_4$ (4.2 g) for 12 h until pH 5.6 and dialysed. *N-Carboxymethyl chitosan* was prepared from shrimp chitosan by reductive amination of glyoxylic acid (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982).

Menadione and coenzyme Q_3 were purchased from Sigma, St Louis, MO, USA. Coenzyme Q_{10} was a kind gift

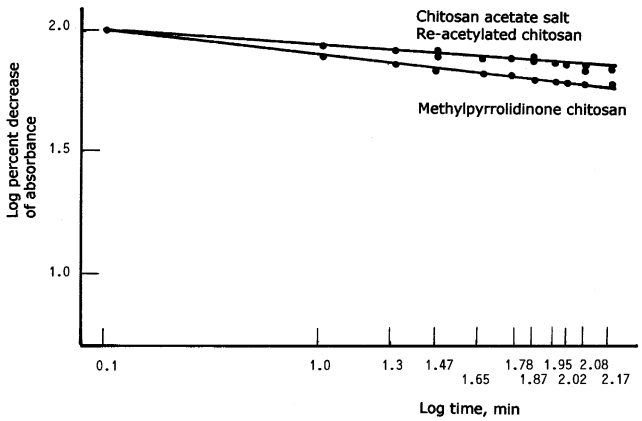

Fig. 1. Double logarithmic plots for the collection of 1,4-naphthoquinone on chitosan acetate salt, re-acetylated chitosan and methylpyrrolidinone chitosan at 20 °C in methanol. The logarithm of the absorbance percent decrease (average of 3 readings) is plotted versus the logarithm of the contact time.

from Kaneka Corp., Osaka, Japan. Other chemicals were from Aldrich, Milano, Italy.

2.2. Film casting

Films were cast from the solution in 7 cm plastic Petri dishes. They were dried at 40 °C and optionally neutralized with NaOH in methanol.

2.3. Instrumental analytical methods

A Perkin–Elmer Spectrum GX FT-IR spectrometer equipped with a Perkin–Elmer Multiscope system infrared microscope (MCT-SL detector) was used to record Attenuated Total Reflectance, ATR, spectra. The microscope was equipped with a movable 75 × 50 mm X–Y stage. In some cases it was necessary to adopt the following procedure: small amounts of the sample, cooled in liquid nitrogen, were ground with KBr and the spectra were obtained by using a Spectra Tech. Diffuse Reflectance (DRIFT) accessory. In both cases, the spectral resolution was 4 cm^{-1}. The absorption spectra were the results of 16 scans. Treatments of the data were achieved with a Perkin–Elmer Spectrum and with a Grams/32 Galactic Corp. software packages.

For contact angle measurements, each chitosan film was assembled in a sandwich of cardboard and filter paper. Such sandwiches were plastified to impart rigidity to the film; the test surface was exposed by cutting away the paper in the area of interest. The films used in the experiments presented different surface roughness. The contact angles, θ, were measured by using 1% NaCl aqueous solution at room temperature. The drop shape was obtained by positioning a light source above the drop and the pictures were taken using a digital camera (Sigma F4-5.6 lens 70–300 mm, with bellows focussing attachment PB-6). The contact angle was obtained according to Muzzarelli, Ilari, Xia, Pinotti, and Tomasetti (1994) by measuring the height h and the width d of the drop, with the formula $\theta = 2 \arctan(2\,h/d)$.

A spectrophotometer Beckman DU 640 and a freeze-drier Heto Drywinner operated at −92 °C and 0.2 mPa were used.

3. Results and discussion

All of the white freeze-dried chitosans once contacted with the methanol solutions of 1,4-naphthoquinone or menadione turned yellow or orange and progressively assumed violet or brown colors in a matter of hours. Their high reactivity was remarked even under unfavourable conditions such as the following: a suspension of chitosan powder in methanol was mixed with a suspension of 1,4-naphthoquinone in acetic acid: an exothermic reaction took

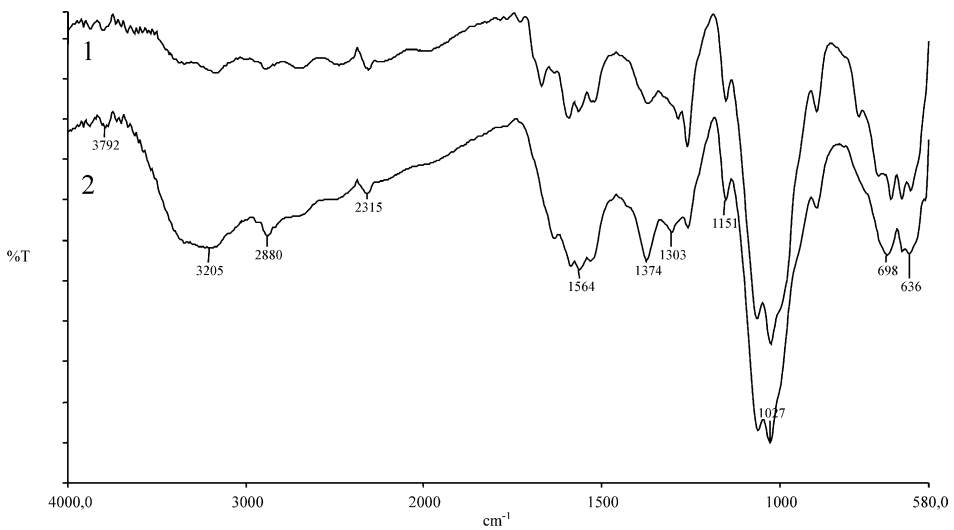

Fig. 2. Micro ATR infrared spectra for the 1.4-naphtho-quinonetreated chitosans, Upper spectrum, dialysed chitosan; lower spectrum, MP chitosan. The main spectral features of chitosans in the range 1700–1500 cm^{-1} and 1400–1300 cm^{-1} are hardly recognisable.

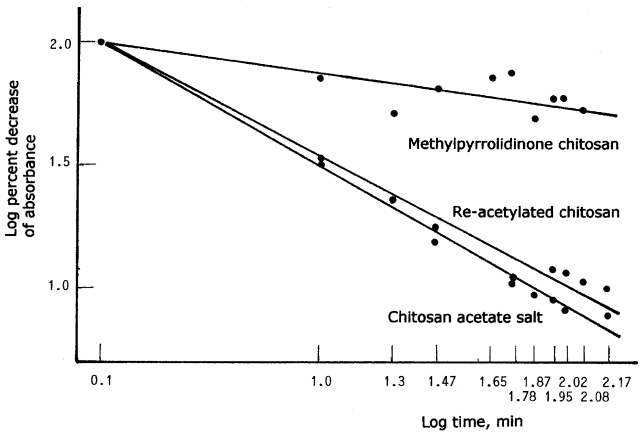

Fig. 3. Double logarithmic plots for the collection of menadione on chitosan acetate salt, re-acetylated chitosan and methylpyrrolidinone chitosan at 20 °C in methanol. The logarithm of the absorbance percent decrease (average of 3 readings) is plotted versus the logarithm of the contact time.

place leading to the formation of a green gel that turned brown overnight. The reaction of these quinones with precipitated chitosan prevented any further measurement by spectrophotometry, due to the fact that both the chitosan and the supernatant methanol solution turned dark brown. Therefore the instrumental measurements were made with only four of the five chitosans prepared.

3.1. 1,4-Naphthoquinone

The UV–VIS spectrum of 1,4-naphthoquinone showed two bands, the second of which at 331 nm was well suited for quantitative determinations, as shown by the linearity of the calibration curve. At this wavelength, the 1,4-naphthoquinone concentration was measured at fixed times of contact with chitosan. The initial 1,4-naphthoquinone concentration was 4 g/l; the methanolic solution (50 ml) was contacted with 0.2 g of freeze-dried material.

The results indicated that MP-chitosan collected approximately 100 mg of 1,4-naphthoquinone, while the chitosan acetate salt collected 84 mg and the re-acetylated chitosan 92 mg. Because the 1,4-naphthoquinone initially present amounted to 200 mg, these chitosans collected one third to one half of the available 1,4-naphthoquinone under the said conditions. This corresponded roughly to a molar ratio amine/quinone = 2, that reasonably indicates saturation of the chitosan by the 1,4-naphthoquinone within 12 h.

Fig. 1 contains the double logarithmic presentation of the kinetic data obtained by spectrophotometry with 1,4-naphthoquinone. Notwithstanding the impressive colour change taking place since the early contact time, the slopes of the curves in the double log presentation were small, indicative of a relatively slow kinetics (slope −13.7 for chitosan acetate and re-acetylated chitosan; −8.8 for MP-chitosan for the initial 150 min).

The linear plot of the same data in fact indicated that an asymptotic value was not reached in the 4 h period of measurement. The differences among the three chitosans were small, and actually no significant difference was noticed between chitosan acetate salt and re-acetylated chitosan.

Chemical alterations of the chitosans subsequent to contact with quinones were easily detected by infrared spectrometry. The collection of 1,4-naphthoquinone on the chitosans brought about impressive physical changes: the colour turned dark brown and the infrared spectra were heavily altered (Fig. 2). Some of the original spectral features of chitosans were lost particularly in the regions 1600–1500 and 1400–1300 cm^{-1}, as a consequence of

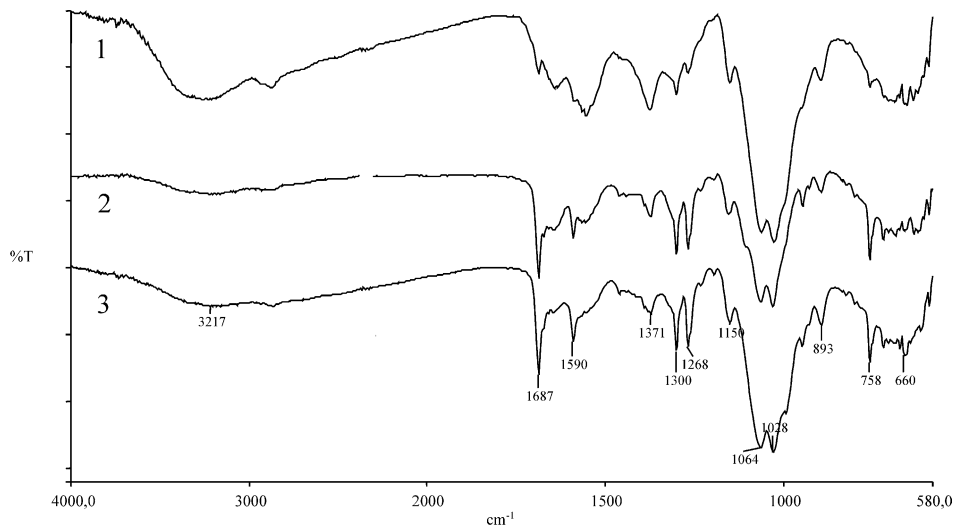

Fig. 4. Micro ATR infrared spectra for menadione-treated chitosans. Spectrum 1 methylpyrrolidinone chitosan, spectrum 2 re-acetylated chitosan, and spectrum 3 chitosan acetate salt. The alterations introduced by adsorbed menadione (whose typical bands are evident) are less remarkable in methylpyrrolidinone chitosan, in agreement with the smaller slope of the relevant collection curve.

Table 1
Contact angles measured for various plain or quinone-treated chitosan films. Aqueous NaCl (9 g/l)

Film	Contact angle(°)
Chitosan + 1,4-naphthoquinone	86.0
Reference plain chitosan	64.1
Chitosan + menadione	57.5
Reference chitosan (NaOH-washed)	40.5

chemical alterations (for example, compare the spectrum of MP-chitosan in Fig. 6 of Muzzarelli, Ilari, and Tomasetti (1993), with spectrum 2 in Fig. 2 of this work). The very sharp bands of 1,4-naphthoquinone at 1659, 1586, 1330, 1200 and 769 cm^{-1} were no longer identifiable in the reaction products, thus indicating that complex reactions took place between the chitosans and 1,4-naphthoquinone.

3.2. Menadione

A quite different situation was found for menadione (Fig. 3), whose kinetics indicate a prompt collection by chitosan acetate salt (slope −1.78) and re-acetylated chitosan (slope −1.93), while the MP-chitosan curve had a small slope (−7.5). In the case of menadione, the chitosans in the freeze-dried form immediately assumed intense colours, such as noisette and brown.

All spectra for menadione-treated chitosans exhibited new bands, in particular at 1687, 1590, 1371, 1300, 1268, 893 and 758 cm^{-1}, all assigned to menadione, as shown in Fig. 4. Because the menadione typical bands were clearly recognized in the products, the collection mechanism for menadione differed substantially from that of 1,4-naphthoquinone.

3.3. Chitosan films

When chitosans are in film form, the contact surface becomes very small compared to the surface of the freeze-dried materials, and therefore the rate of collection sharply decreases. This permits a fine regulation of the degree of substitution because the relatively long contact time would be precisely measurable.

Thin chitosan films treated with 1,4-naphthoquinone were observed directly after mounting them on a frame and positioning them on the infrared beam path. Small quantities of quinones introduced modest alterations in the spectra, which however became appreciable in the derivative spectra, particularly in the range of 1650 cm^{-1}. The chemical modification introduced by 1,4-naphthoquinone and menadione in the chitosan film surface, as revealed by optical instruments, was large enough to alter the shape of a drop of saline deposited there. For 1,4-naphthoquinone-treated chitosan film the contact angle was 86.0° rather than 64.1 for the control untreated film. For menadione-treated chitosan films previously washed with alcoholic NaOH and alcohol, the contact angle was 57.5 instead of 40.5 for control (Table 1).

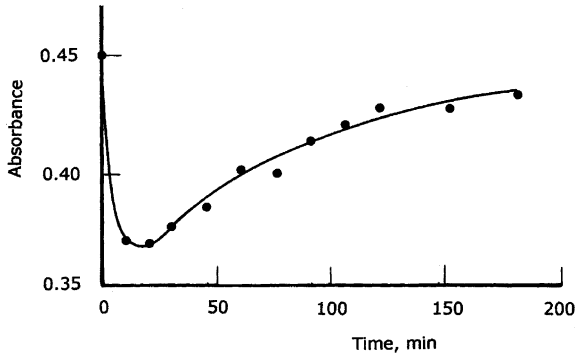

Fig. 5. Linear plot of the absorbance percent decrease versus time for methylpyrrolidinone chitosan and plumbagin at 20 °C in methanol.

3.4. Plumbagin

Plumbagin solutions (200 mg in 20 ml methanol) were used to obtain collection data for the freeze-dried chitosans (200 mg) via spectrophotometric readings at 530 nm. In all cases, the chitosans turned dark colored, but the collection capacity did not seem to be high, and the reactivity rather limited. In fact, after the initial 10 min of contact, the plumbagin concentration in the supernatant had a small but significant increase, possibly due to release of adsorbed plumbagin. Typical results are shown in Fig. 5 for MP-chitosan.

3.5. CoQ_{10} and CoQ_3

The materials under study were also contacted with methanol solutions of either CoQ_{10}, or CoQ_3 under the same experimental conditions. Spectrophotometric evidence indicated that these quinones do not react with the chitosans tested under the present conditions. The spectrophotometric readings taken over a 4 h contact period indicated that no decrease of CoQ_{10} and CoQ_3 concentration took place, and all the five chitosan freeze-dried materials listed under 2.1 remained white.

4. Conclusions

The quinones taken into consideration in this paper, all endowed with biological relevance, have been tested with chitosans for the first time. Reactivity can be ascribed, in general, to the chemical features of the different quinones, to the chemical functions present in the chitosans, and to their physical form. For the reactive quinones, generally a biphasic pattern was suggested by the rapid initial course of the reaction (initial 150 min) followed by a long lasting slow phase (observed up to 4 h).

The lack of reactivity of CoQ_{10} and CoQ_3 was unexpected, and did not depend on the isoprenoid chain length. The limited reactivity of plumbagin (as well as 1,2-naphthoquinone) was also noted.

N-carboxymethyl chitosan was recently formulated in tooth pastes, mouth rinses, artificial saliva and other articles of interest in dentistry (Muzzarelli, Cucchiara, & Muzzarelli, 2002). Because the above described lack of reactivity between *N*-carboxymethyl chitosan and CoQ_{10} allows the co-existence of the two compounds, which will retain their respective biological efficacy when formulated together, *N*-carboxymethyl chitosan and CoQ_{10} are being co-formulated in different products, for topical application, in the dentistry and cosmetic areas, with the intention of taking advantage simultaneously of the antioxidant role of ubiquinone and the reparative action of the water-soluble modified chitosan. Ubiquinone could be incorporated into the formulations as an ethanol solution at the time of the addition of aromas.

On the other hand, menadione-treated chitosans might have enhanced hemostatic activity, and work in progress will provide experimental evidence of their efficacy.

Acknowledgements

Thanks are due to Mrs Carla Conti, Department of Materials Sciences, for recording micro ATR infrared spectra, to Dr Rudolf Huebner, Department of Mechanics, for contact angle measurements, and to Mrs Maria Weckx, Institute of Biochemistry, for retrieving and handling the bibliography.

References

Chen, T., Kumar, G., Harris, M. T., Smith, P. J., & Payne, G. F. (2000). Enzymatic grafting of hexyloxyphenol onto chitosan to alter surface and rheological properties. *Biotechnology and Bioengineering*, *70*, 564–573.

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., DeBenedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly(ethyleneglycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*, 201–206.

Edwards, W., Leukes, W. D., Rose, P. D., & Burton, S. G. (1999). Immobilization of polyphenol oxidase on chitosan-coated polysulphone capillary membranes for improved phenolic effluent bioremediation. *Enzyme and Microbial Technology*, *25*, 769–773.

Ernster, L., & Beyer, R. E. (1991). Antioxidant functions of Coenzyme CoQ_{10} some biochemical and pathophysiological implications. *Biomedical and Clinical Aspects of CoQ*, *6*, 45–58.

Harding, S. E. (1997). Characterization of chitosan–mucin complexes by sedimentation velocity analytical ultracentrifugation. In R. A. A. Muzzarelli, & M. G. Peter (Eds.), *Chitin Handbook* (pp. 457–466). Italy: Atec.

Holl, M. S., Schaefer, J., Goldberg, W. M., Kramer, K. J., Morgan, T. D., & Hopkins, T. L. (1992). Comparison of black coral skeleton and insect cuticle by a comparison of C13 NMR and chemical analyses. *Archives Biochemistry and Biophysics*, *292*, 107–111.

Hoppe, U., Bergemann, J., Diembeck, W., Ennen, J., Ghola, S., et al. (1999). Coenzyme Q_{10}, a cutaneous antioxidant and energizer. *BioFactors*, *9*, 371–378.

Hua, J., Sakamoto, K., & Nagaoka, I. (2002). Inhibitory actions of glucosamine, a therapeutic agent for osteoarthritis, on the functions of neutrophils. *Journal of Leukocyte Biology*, *71*, 632–640.

Kishi, T., Takahashi, T., Usui, A., Hashizume, N., & Okamoto, T. (1999). Cystolic NADPH-UQ reductase, the enzyme responsible for cellular ubiquinone redox cycle as an endogenous antioxidant in the rat liver. *BioFactors*, *9*, 189–197.

Klokkevold, P. R., Fukayama, H., Sung, E. C., & Bertolami, C. N. (1999). The effect of chitosan (poly-*N*-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. *Journal of Oral and Maxillofacial Surgery*, *57*, 49–52.

Kulling, D., Vournakis, J. N., Woo, S., Demcheva, M. V. B., Tagge, D. U., Rios, G., & Haves, R. H. (1999). Endoscopic injection of bleeding esophageal varices with a poly-*N*-acetylglucosamine gel formulation in the canine portal hypertension model. *Gastrointestinal Endoscopy*, *49*, 764–771.

Kumar, G., Bristow, J. F., Smith, P. J., & Payne, G. F. (2000). Enzymatic gelation of the natural polymer chitosan. *Polymer*, *41*, 2157–2168.

Kumar, G., Smith, P. J., & Payne, G. F. (1999). Enzymatic grafting of a natural product onto chitosan to confer water solubility under basic conditions. *Biotechnology and Bioengineering*, *63*, 154–165.

Mancini, S., Muzzarelli, R. A. A., & Mancini, St. (2000). *Trattato di Flebologia, Utet, Italy*, *2*, 671–682.

Mitchell, P. (1976). Possible molecular mechanisms of the protonmotive function of cytochrome systems. *Journal of Theoretical Biology*, *62*, 327–367.

Muzzarelli, R. A. A. (1997). Human enzymatic activities related to the therapeutical administration of chitin derivatives. *Cellular and Molecular Life Science*, *53*, 131–140.

Muzzarelli, R. A. A., Biagini, G., Bellardini, M., Simonelli, L., Castaldini, C., & Fratto, G. (1993). Osteoconduction exerted by *N*-methylpyrrolidinone chitosan used in dental surgery. *Biomaterials*, *14*, 39–43.

Muzzarelli, R. A. A., Cucchiara, M., & Muzzarelli, C. (2002). *N*-Carboxymethyl chitosan in innovative cosmeceutical products. *Journal of Applied Cosmetology*, 201–208.

Muzzarelli, R. A. A., Ilari, P., & Tomasetti, M. (1993). Preparation and characteristic properties of 5-methyl pyrrolidinone chitosan. *Carbohydrate Polymers*, *20*, 99–106.

Muzzarelli, R. A. A., Ilari, P., Xia, W., Pinotti, M., & Tomasetti, M. (1994). Tyrosinase-mediated quinone tanning of chitinous materials. *Carbohydrate Polymers*, *24*, 294–300.

Muzzarelli, R. A. A., Tanfani, F., Emanuelli, M., & Mariotti, S. (1982). *N*-Carboxymethylidene chitosans and *N*-Carboxymethyl chitosans: novel chelating polyampholytes obtained from chitosans glyoxylate. *Carbohydrate Research*, *107*, 199–219.

Okamoto, Y., Watanabe, M., Miyatake, K., Morimoto, M., Shigemasa, Y., & Minami, S. (2002). Effects of chitin/chitosan and their oligomers/monomers on migrations of fibroblasts and vascular endothelium. *Biomaterials*, *23*, 1975–1979.

Passi, S., De Pita, O., Puddu, P., & Littarru, G. P. (2002). Lipophilic antioxidants in human sebum and aging. *Free Radical Research*, *36*, 471–478.

Patel, A. R., Sun, W. Q., & Payne, G. F. (1994). Tyrosinase reaction/chitosan adsorption: potential for removing polymerization storage inhibitors. *Industrial Engineering Chemistry Research*, *33*, 2168–2173.

Payne, G. F., & Sun, W. Q. (1994). Tyrosinase reaction and subsequent chitosan adsorption for selective removal of a contaminant from a fermentation recycle stream. *Applied Environmental Microbiology*, 397–401.

Payne, G. F., Sun, W. Q., & Sohrabi, A. (1992). Tyrosinase reaction/chitosan adsorption for selectively removing phenols from aqueous mixtures. *Biotechnology and Bioengineering*, *40*, 1011–1018.

Shao, L., Kumar, G., Lenhart, J. L., Smith, P. J., & Payne, G. F. (1999). Enzymatic modification of the synthetic polymer polyhydroxystyrene. *Enzyme and Microbial Technology*, *25*, 660–668.

Stocker, R., Bowry, V. W., & Frei, B. (1991). Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid preoxidation than does α-tocopherol. *Proceedings National Academy of Sciences USA*, *88*, 1646–1650.

Sugumaran, M. (1988). Molecular mechanisms for cuticular sclerotization. *Advances in Insect Physiology*, *21*, 179–231.

Sun, W. Q., & Payne, G. F. (1996). Tyrosinase-containing chitosan gels: a combined catalyst and sorbent for selective phenol removal. *Biotechnology and Bioengineering*, *51*, 79–86.

Sun, W. Q., Payne, G. F., Moas, M. S. G. L., Chu, J. H., & Wallace, K. K. (1992). Tyrosinase reaction/chitosan adsorption for removing phenols from wastewater. *Biotechnology Progress*, *8*, 179–186.

Vachoud, L., Chen, T. H., Payne, G. F., & Vasquez-Duhalt, R. (2001). Peroxidase catalyzed grafting of gallate esters onto the polysaccharide chitosan. *Enzyme and Microbial Technology*, *29*, 380–385.

Wu, L. Q., Chen, T. H., Wallace, K. K., Vazquez-Duhalt, R., & Payne, G. F. (2001). Enzymatic coupling of phenol vapors onto chitosan. *Biotechnology and Bioengineering*, *76*, 325–332.