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ABSTRACT

We studied the application of unilamellar liposomes of a defined size (200 nm) containing cholesterol as vehicles for oxidative reagents in wool chlorination. To this end, we first studied the interaction between liposomes and chlorine in order to determine the physicochemical stability of these systems in the presence of this oxidative agent. We assessed physical stability by measuring both the mean vesicle size distribution of the vesicle suspensions and the changes in the ahsorbance of these systems, which are directly related to the aggregation or solubilization of liposomes. Our stndy of chemical stability was based on the lipid peroxidation index of liposomes at different chlorine concentrations at pH value 6.5. As regards the oxidative effects caused by the chlorine treatments of wool applied directly or by means of liposomes at pH 1.5, we investigated the extent of cysteic acid formation groups in wool fibers. Increasing amounts of cholesterol in the id ilay s of liposomes enhance both the physicochemical stability of these systems and the inhibitory ability of cysteic acid formation when samples have been treated with chlorine-liposome systems.

The reaction of keratin fibers with aqueous chlorine solutions has been widely investigated, with special attention paid to the quantitative and qualitative aspects of the oxidizing reactions that lead to both chemical and physical modifications of the fiber [15, 22, 28]. The chlorination process preferentially oxidiies cystine residues to cysteic acid residues; in acidic solutions, the oxidation occurs by means of intermediate oxidation products. In general terms, there is a tendency in technological processes to either suhstitute the chlorination of the fihers by other oxidative processes with lesser degradative and more homogeneous effects, or even to avoid the oxidative pretreatment altogether [7, 9, 21].

Phospholipid vesicles or liposomes are lipid-water systems that have come into widespread use as simplified models of different biological membranes and as delivery systems where encapsulation and protection of substancesare required for hydrophilicand lipophilic structures [3, 4, 14, 27].

Although initially slow to exploit the technology of liposomes, the textile industry has now produced a wide vanety ofinnovations using the basic principles of targeting, slow release, and protection of sensitive chemicals, principally in dyeing and finishing [6, 17].

Including cholesterol in bilayer membranes has a condensing effect and tends to retard protein penetration [10]. The presence of this component is also a very important factor for obtaining liposome formulations that are both stable in biological environments and suitable as chemical camers [20]. Furthermore, the liposome-cellfusion is based largely on the apparent uptake of liposomal lipids or their contents by the cells [2, 18].

Previously, we reported using phosphatidylcholine liposomes as a vehicle for aqueous chlorine solutions in wool chlorination processes [5]. In this work, bearing in mind that cholesterol is one of the main components of the internal lipids of wool [23], we have studied the effects caused by including this component in lipid bilayers to obtain improved liposome technological applications. To this end, we have investigated the physiwchemical stability of phosphatidylcholine/cholesterol liposomes at different lipid concentrations (PC : CH 10:0, 9:1, and 8:2 molar ratios) in the presence of different aqueous solutions of sodium hypochlorite at pH 6.5 during and after preparation. Furthermore, we have evaluated the use of these liposomes as camers of chlorine aqueous solutions to wool fibersat different chlorine concentrations and acid pH to obtain improved chlorination. For this purpose, we studied the cysteic acid content of the chlorinated wool fihers, treated directly or with liposomes, in a lipid concentration range between 0 and 25 mmol.

Experimental

MATERIALS

Australian Merino wool 64's samples were Soxhlet extracted for 2 hours with methylene chloride and rinsed with water purified by the Milli-Ro system (Millipore) to remove all contaminating substances. Samples were then dried at room temperature.

Phosphatidylcholine was purified from egg lecithin (Merck) according to the method of Singleton [24] and shown to be pure by thin-layer chromatography. Cholesterol was purchased from Sigma. The sodium hypochlorite solution was an 8% w/v active chlorine solution obtained from Carlo Erba. The other chemicals used throughout were of reagent grade quality. Polycarbonate membranes of 200, 400, and 800 nm and membrane holders used for liposome extrusion were purchased from Nucleopore.

METHODS

Unilamellar vesicle suspensions (LUV) of defined vesicle size (200 nm) and different lipid concentrations (1 to 25 mmol) containing diverse concentrations of sodium hypochlorite aqueous solutions were prepared essentially following a method described by Bangham [1].

A lipid film was formed by removing the organic solvent under nitrogen atmosphere and low vacuum (350 mm Hg) by rotatory evaporation from a chloroform solution of egg phosphatidylcholine/cholesterol (10:0, 9:1, and 8:2 molar ratios). An aqueous phase containing KCl (200 mmol) and different concentrations of sodium hypochlorite to be trapped in the lipid film was then added. The solutions were stirred by hand to deliver the lipid from the walls of the flask and to disperse large lipid aggregates; glass beads were added to facilitate dispersions. The resulting milky suspensions were vortexed for 5 minutes. The liposome suspensions were extruded through 800,400, and 200 nm polycarbonate membranes to obtain a uniform size distribution [26], and the pH value was adjusted to 6.5 with 4 N hydrochloric acid. Finally, the chlorine content in the liposome suspensions was determined [16].

Merino wool fibers (1 g) were treated with LUV liposomes (70 ml) freshly prepared at different lipid concentrations (1 to 25 mmol) containing three different concentrations of sodium hypochlorite solutions (0.07, 0.14, and 0.28% Cl_2 weight/volume), after adjusting the liposome suspension to pH = 1.5 with hydrochloric acid (buffer KCl/HCl). The treatments were done for 15 minutes at 20°C and stirred constantly in a closed bath. Samples were then rinsed with water and treated with sodium metabisulphite (1%)at a bath ratio of 1:30 for 5 minutes to remove the excess oxidative reagent. The chlorinated wool samples were then rinsed in water purified by the Milli-Ro system and dried at room temperature.

CHARACTERIZATION AND STABILITY OF LIPOSOME PREPARATIONS

The mean particle size and polydispersity of the LUV liposome preparations were determined by a Photon Correlator spectrometer (Malvern Autosizer 4700c PS/MV). Samples were adjusted to the appropriate concentration range with a 200 mmol KCl aqueous solution at pH 6.5. The measurements were made at 25°C, with a detection angle of 90".

The aggregation state of the vesicles was estimated as a measurement of the physical stability of the liposome suspensions. This was done by monitoring the absorbance variations of the liposome suspension (5 mmol lipids) using a Shimadzu UV spectrophotometer (λ = 500 nm, cell length = 0.2 cm), and by measuring the variation of the mean vesicle size distribution of liposome suspensions as a function of time.

According to Klein [12], the ratio of absorbance at 233 and 215 nm can be taken as a measurement of lipid peroxidation. In this work, we determined the presence of conjugaied dienes after adding 0.1 ml of a specific liposome preparation (20 mmol of lipids in a 200 mmol KCl aqueous solution) to 3.0 ml of absolute ethanol, and measuring absorbance in a 1 cm cell length at 233 and 215 nm, using a Shimadzu UV-240 spectrophotometer.

The concentration of active chlorine in the liposome suspensions was determined by using the iodometnc method [16]. The oxidation state of the wool fibers was determined by measuring the cysteic acid content of chlorinated wool samples using the IWTO method [25].

Results

AGGREGATION MEASUREMENTS

Changes in liposome size due to aggregation or solubilization can be followed up by measuring both changes in absorbance and mean vesicle size distribution of these systems using spectrophotometric and quasi-elastic light scattering methods [4, 11], respectively.

The chlorine concentration for each liposome suspension is given as the molar ratio between both chlorine and lipid components, which in this paper will be

defined as K. Figure *1* shows optical density variations versus time of chlorine-liposome suspensions at pH *6.5* with different molar ratios *(K)*. The optical density of liposome suspensions decreases linearly with time, being especially pronounced when the K values are higher than *40 (20%* absorbance decrease after *4–6* hours for *K = 40)*. For *K* values lower than *5*, the changes in absorbance of these systems versus time are clearly reduced. Thus, for K = *5.0,* there is only a *30%* decrease in absorbance following *24* hours of liposome preparation. Furthermore, increasing amounts of cholesterol in lipid bilayers results in an increased stability of these structures to the independent aggregation of the K chlorine/lipid molar ratios. Thus, liposome suspensions containing cholesterol (PC : CH *8:2* molar ratio) show only a *20%* decrease after *24* hours.

The result of monitoring the variation of mean vesicle size over time of a liposome suspension for K = *5.0* is indicated in Table I. Regarding the data, a low decrease in the mean vesicle size occurs with time, the polydispersity index remaining below *0.25 24* hours after preparation of the liposomal suspensions. It is noteworthy that including increasing amounts of cholesterol in liposomes increases the stability of these structures with respect to aggregation, both the mean particle size distribution and the polydispersity indexes being enhanced. The results shown in Table I are in agreement with those given in Figure 1.

LIPID PEROXIDATION

We detected the formation of conjugated dienes in unsaturated fatty acids by measuring the UV adsorption of liposome suspensions at *233* and 215 nm. In accordance with Klein's suggestion [12], we used 215 nm as a reference wavelength, the lowest wavelength where the adsorption depended linearly on the amount of lipid used and was independent of the degree of

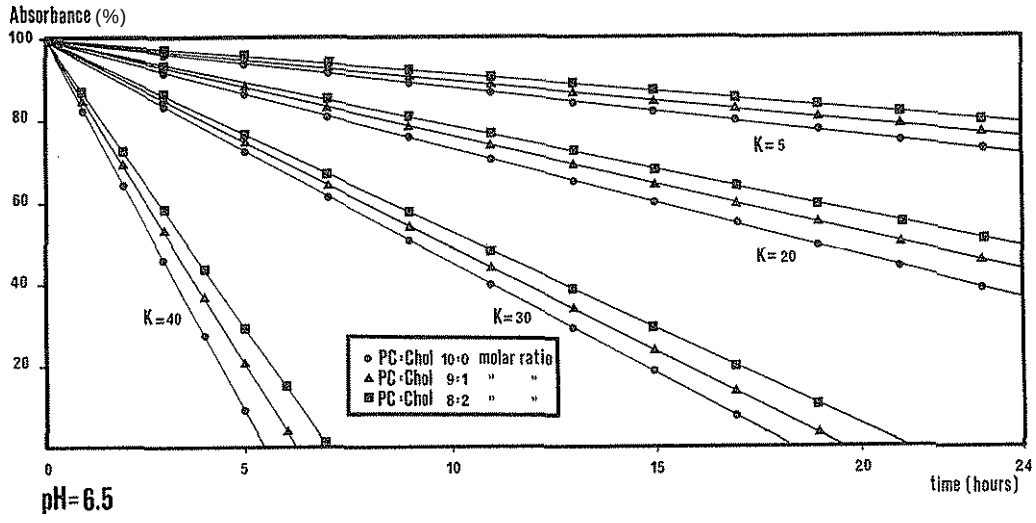

FIGURE 1. Absorbance of LUV chlorine-liposome suspensions containing increasing amounts of cholesterol in the lipid bilayers (PC : CH 10:0, 9:1, and 8:2 molar ratios) at different chlorine phospholipid molar ratios (*K*), al pH 6.5, versus time.

TABLE 1. Variations of mean vesicle size and polydispersity of chlorine liposomes at pH 6.5 containing increasing amounts of cholesterol in the lipid bilayers (PCCH 10:0, 9:1, and 8:2 molar ratios) at a chlorine lipid molar ratio of K = 5.0, over time.

Lipid composition	Time, hours															
	1		2		4		8		12		16		20		24	
	A[a]	B[b]	A	B	A	B	A	B	A	B	A	B	A	B	A	B
PC:CH 10:0	202	0.113	203	0.118	198	0.126	189	0.140	188	0.148	182	0.158	178	0.175	170	0.194
PC:CH 9:1	200	0.112	201	0.115	199	0.117	197	0.124	193	0.138	190	0.146	184	0.156	180	0.185
PCCH 8:2	201	0.110	200	0.112	200	0.114	199	0.116	196	0.123	194	0.132	187	0.147	185	0.176

[a] A = mean vesicle sire. nm. [b] B = Polydispersity index.

peroxidation until an $A_{233\ nm}/A_{215\ nm}$ of 0.8 [13]. The ratio of these absorptions is defined as the "oxidation index" and shown in Figure 2. In this figure, the oxidation index of LUV liposome suspensions containing different concentrations of sodium hypochlorite solutions (lipid concentration 20 mmol) at pH 6.5 is plotted versus time. The oxidation index increases linearly with time for each molar ratio of chlorine/lipid (K) for the liposome suspension investigated. Thus, for a K value of 20, the lipid peroxidation increases rapidly (oxidation index = 0.8 after 6–7 hours of treatment), and for low K values the increase in peroxidation is not very rapid. Approximately 21 hours are needed to detect an oxidation index value of around 0.8 for K = 5.0. Moreover, in all cases, liposomes containing increasing amounts of cholesterol appear to be more resistant to the lipid peroxidation, especially for K molar ratios lower than 5.

In general terms, we could assign the importance of the presence of the sodium hypochlorite solutions trapped in liposome suspensions in relation to the physicochemical stability of liposomal structures. In this connection, and given that hypochlorous acid predominates at pH 6.5, we have concluded from the results reported above that the physicochemical stability of these systems with time depends directly on the molar ratios existing between the oxidizing agents and the lipids. Only at Kvalues lower than 2.0 is there physicochemical stability during a time period exceeding 24 hours at pH 6.5. Note that the presence of increasing amounts of cholesterol in the lipid bilayers (molar ratios 9:1 and 8:2) results in an increased stability of the chlorine-liposome systems versus lipid peroxidation and vesicle aggregation.

TREATMENTS OF SAMPLES WITH CHLORINE LIPOSOMES

Wool fibers were immersed in chlorine-liposome suspensions prepared at pH 6.5, and subsequently adjusted to pH 1.5, according to the experimental specifications described above. Figure 3 shows the cysteic acid content (μmols/g wool) of wool fiber samples treated with chlorine liposomes at three different chlorine concentrations (0.07, 0.14, and 28% w/v) versus liposome lipid concentrations. Three different bilayer lipid compositions were used (PC : CH 10:0, 9:1, and 8:2 molar ratios).

A clear inhibition (50% approximately) in cysteic acid formation occurred for the three chlorine concentrations investigated. This inhibition seems to be closely connected to the molar ratio values (K) between both chlorine and lipid liposome components. Moreover, the presence of increased amounts of cholesterol in lipid bilayers results in an increased capacity of liposomes to inhibit wool oxidation. Thus, in treatments of wool fibers at 0.28% chlorine concentration (curve A), liposomes containing the highest concentration of cholesterol (PC : CH 8:2 molar ratio) show inhibition of cysteic acid formation from a molar ratio value (K) corresponding to 12.9, whereas liposomes withont cholesterol show this capacity from a lesser K value (K

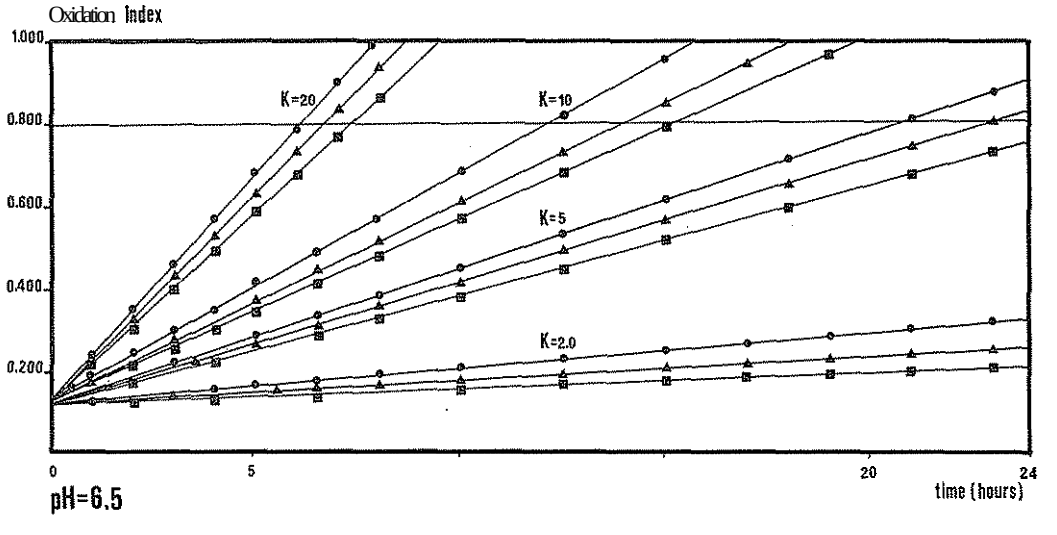

FIGURE 2. Oxidation index of LUV chlorine-liposome suspensions containing increasing amounts of cholesterol in the lipid bilayers (PC: CH 10:0, 9:1, and 8:2 molar ratios) at different chlorine phospholipid molar ratios (K), at pH 6.5, versus time.

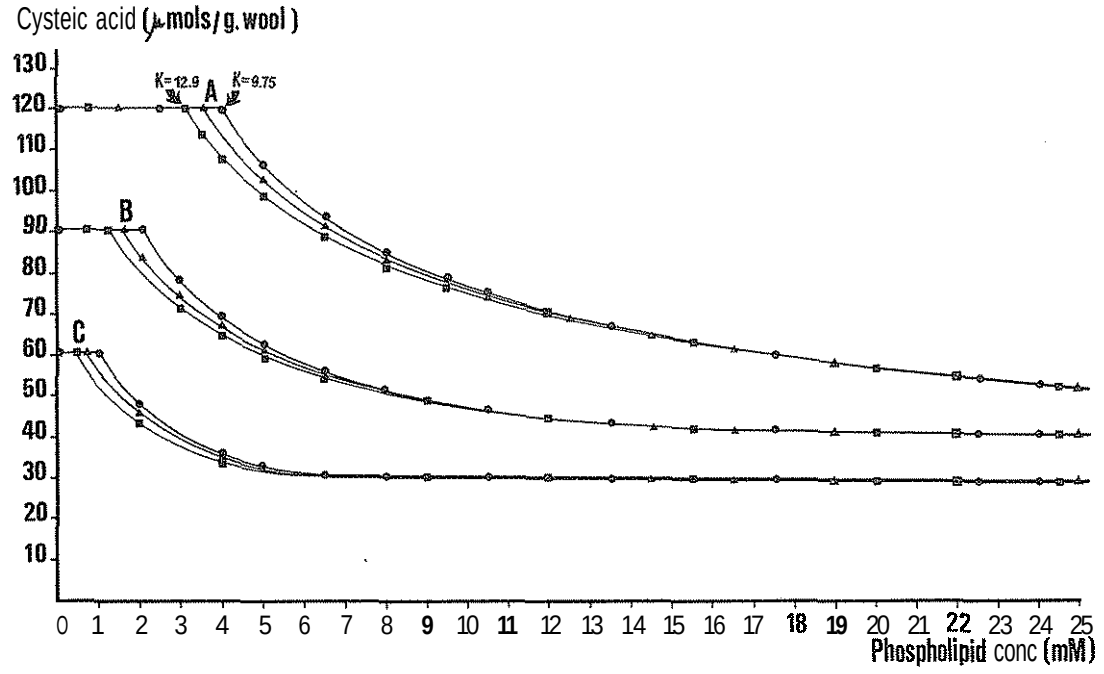

FIGURE 3. Cysteic acid content of wool fibers treated with chlorine-liposome suspensions containing increasing amounts of cholesterol in the lipid bilayers (PC : CH 10:0, 9:1, and 8:2 molar ratios) at three chlorine concentration levels (A = 0.28%, B = 0.14%, and C = 0.07%, w/v), versus phospholipidic concentration in liposomes.

= 9.75). As a consequence, in the first case low liposome lipid concentrations are needed to initiate the inhibitory process. There were similar tendencies for all the chlorine concentrations studied (curves B and C corresponding to the 0.14 and 0.07% chlorine concentrations, respectively). Furthermore, the highest inhibiting power of cysteic acid formation corresponds, in all cases, to the *K* values of about 2.0.

We obtained the curves in Figure 4 by plotting the chlorine/lipid molar ratio values *(K)* necessary to initiate the inhibiting capacity of cysteic acid formation versus chlorine concentration in liposomes. Note that bilayer structures containing the highest cholesterol concentration (PC : CH 8:2 molar ratio) inhibit, in all cases, the cysteic acid formation from higher molar ratio values *(K)*. However, this capacity decreases as the chlorine concentration in liposomes increases.

As regards the physicochemical stability of the chlorine liposomes at pH 1.5 during wool treatments, the oxidation index value rose rapidly and reached values exceeding 0.8. Moreover, the mean vesicle size distribution during these applications showed a rapid degradation of the lipid bilayers. Both effects occurred as a consequence of the presence of free chlorine in the liposome suspensions at this pH.

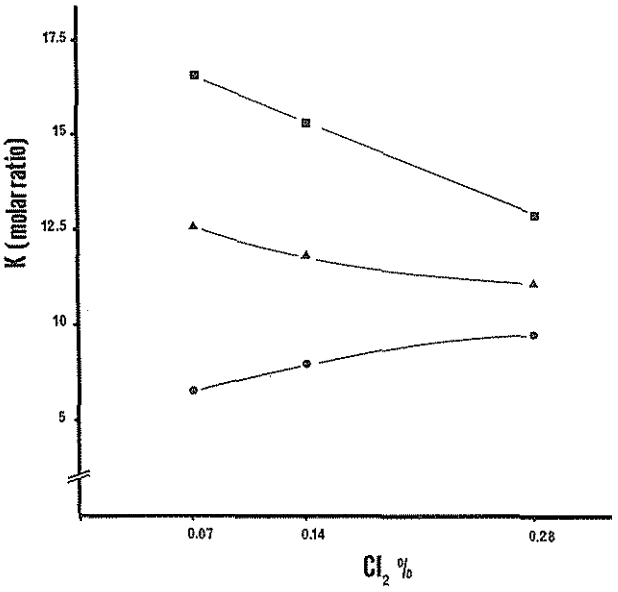

FIGURE 4. Chlorine/lipid molar ratios (*K*) necessary to initiate the inhibiting capacity of cysteic acid formation when wool fibers are treated with chlorine-liposome systems containing different proportions of cholesterol in the lipid bilayer (PC : CH 10:0, 9:1, and 8:2 molar ratios) versus chlorine concentration in liposomes (%).

This paper seeks to extend our investigations of liposomes in wool chlorination [5] by studying the influence of the lipid bilayer compositions on the stability and applications of these systems. The inclusion of cholesterol in liposome bilayers is a very important factor for obtaining liposome formulations that are more stable with respect to the chlorine aqueous solutions at neutral pH values and are also suitable as chlorine camers in wool chlorination processes as well as olher stages of wool processing.

Particularly during the conventional oxidative process, there is a strong affinity between active chlonne and wool fibers, which determiues a fast and uneven reaction on the active sites of the fibers. The oxidative irregularity could influence other parts of the industrial processing of wool (shnnkproofing, dyeing, etc.). Using liposomes in wool chlorination, especially those containing cholesterol in the lipid bilayers, could inhibit excessive fiber oxidation, resulting in a more regular oxidative attack on the surface of the fibers and improved performance during the last stages of processing.

Conclusions

From the work reported here, we have concluded that the presence of cholesterol in phospholipid bilayer liposomes can be considered suitable for enhancing both the stability of these systems and the applications of these structures in wool chlorination.

The results from the physicochemical stability of chlonne liposome suspensions at pH 6.5 show that for chlorine/lipid molar ratio (K) values lower than 2.0, the liposome suspensions are physicochemically stable during a period of 24 hours. Increasing amounts of cholesterol in lipid bilayers increase the physicochemical stability of liposomes, making these structures more resistant to aggregation and lipid peroxidation.

The inhibitory capacity of liposome-clorine systems in cysteic acid formation of treated wool fibers is increased by the presence of cholesterol in lipid bilayers. These improvements are obtained in all the chlorine/lipid molar ratios studied, regardless of the chlorine aqueous concentrations used.

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