# W TO PREPARE LIPOSOMES TO STUDY OXIDATION OF MUSCLE PHOSPHOLIPIDS ?

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## MMARY

spholipids (PL) of pork muscle were used to prepare multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large lamellar vesicles (LUV) which were obtained respectively by vortexing, sonication and extrusion. No quantitative losses in phospholipids re found after liposome preparation. Conjugated dienes and TBA values were always very low after liposome preparation, but these ues were the highest for SUV and the lowest for MLV, the values obtained for LUV being either intermediate (dienes) or equal to SUV BA). After 3 days of storage in the dark at 4°C, TBA and conjugated dienes values were unchanged while they increased slightly after 7 ys of storage. No change in polyunsaturated fatty acids content nor in relative proportions of PL classes was observed in liposomes as npared to PL extracts. Regarding effects of preparation upon muscle PL oxidation, easiness of preparation and distibution of size and mber of bilayer of vesicles, extrusion should be preferred to prepare model systems to study muscle PL oxydation.

## TRODUCTION

spholipids are the major substrate for lipid oxidation in meat and meat products (KELLER and KINSELLA, 1973). If pathways for fatty id oxydation are well known (see the review of FRANKEL, 1982 and the book of CHAN, 1987 for exemple), oxidation kinetics of nplex molecules as phospholipids require more investigation (i.e. : what are the relative influences of polar head and fatty acid moiety of molecules towards oxidation ? ; what are the influences of the physical structure of the lipids and of other constituents ?). To study spholipid oxidation, liposomes are attractive model systems (CHATTERJEE and AGARWAL, 1988). Many methods to prepare osomes have been previously described (LICHTENBERG, 1988). These methods were generally developed on synthetic phospholipids, soybean or egg phosphatidyl-choline. The high content of long chain polyunsaturated fatty acids (PUFA) in muscle phospholipids and ir complex composition (i.e. the presence of ether lipids) required to study specifically the effects of liposome preparation on spholipid degradation and on the properties of the liposomes.

this paper, three methods of liposomes preparation are compared regarding their consequences on phospholipid degradations. These thods were choosen since no additive (as diethylether, ethanol or detergents) was used during liposomes preparation.

## TERIALS and METHODS

**spholipid Preparation** : Intramuscular lipids were extracted from pork *Longissimus dorsi* with 2:1 dichloromethane-methanol (FOLCH al, 1957). Phospholipids were purified on silicic acid column (BORGSTROM, 1952) and stored in dichloromethane at - 80°C until use. o PL extracts were prepared and handled separately (P1 and P2).

**osome preparation** : About 25 mg of phospholipids (9x) in dichloromethane were poured in cap-locked tubes. Solvent was evaporated der $N_2$ blowing and then the tubes were maintained 30 min under $N_2$ to eliminate solvent traces. 10 ml of filtered (0.2µm pore size filter) d degazed cacodylate buffer (0.1M ; pH 5.8 ; 0.05M NaCl) was poured over lipids which were keeped overnight at 4°C in the dark to ure polar head hydratation. Multilamellar vesicles (MLV) were prepared by vortexing phospholipid suspension for 10 min. Small lamellar vesicles (SUV) were obtained by sonication of 3 MLV tubes for 30 min under nitrogen in an ice bath using a 500 W, 20 kHz e sonifier (Sons ans Sonics Inc.) equipped with a titanium microtip (output : 4 ; 40% duty cycle). The suspension was centrifuged at

2500 g for 15 min to remove titanium particles and possible large phospholipid aggregates. Large unilamellar vesicles (LUV) were prep[ared] by extrusion of MLV (3 tubes) through 2 polycarbonate filters as described by HOPE and al. (1985). As plugging occurs during muscle [ ] extrusion, 10 successive extrusions were performed in a following way : 2 extrusions through 0.2 µm filters and 8 through 0.1 µm filter[s] the filters being changed after the first 0.1 µm extrusion.

The nine vesicle suspensions (3 MLV, 3 SUV and 3 LUV) whose final volumes were measured, were poured in 50 ml glass bottles fi[tted] with PTFE lined screwed caps, diluted to 25 ml with cacodylate buffer, oxygenated at room temperature for 10 min using filtered air [and] keeped at 4°C in the dark. Sampling was performed just after liposome preparation and after 3 and 7 days of storage.

***Alterations of phospholipids*** : Thiobarbituric acid (TBA) measurements were performed directly on 3 x 0.5 ml of liposome suspensio[n] (BUEGE and AUST, 1978). TBA values were expressed as absorbance at 532 nm per 1 mg of fatty acids. Conjugated dienes we[re] determined by the ratio $A_{233}/A_{215}$ measured on 2 x 0.25 µl of liposome suspension diluted to 2 ml in ethanol (KLEIN, 1970). Quantification of PUFA, and of total fatty acids and aldehydes were achieved in duplicate on 1 ml of liposomes. 100 µg of heptadecanoi[c] acid was added as internal standard. Then, the samples were extracted by dichloromethane : methanol (2:1 ; V/V ; 0.02% BHT). Fatty aci[ds] and aldehydes were analyzed as methyl esters and dimethyl-acetals (DMA) prepared as described by BERRY *et al.* (1965) by g[as] chromatography. To check repartition of PL classes within liposomes, PL were extracted from 2ml of liposomes by dichloromethan[e :] methanol (2:1; V/V ; 0.02% BHT). Composition in phospholipid classes was determined by HPLC (LESEIGNEUR *et al.*, 1989).

***Physical stability of liposomes*** : It was evaluated by the evolution of the turbidity of the suspensions measured at 350 nm.

## RESULTS

***Phospholipid yield*** : Taking into account liquid handling volume losses, and according to fatty acid and aldehyde determination, no loss [of] phospholipid was detected whatever the method of preparation or the time of storage. Moreover, compared to the composition of the [PL] extracts, no change in fatty acid and aldehydes composition (PUFA : 41 % ; C18:2 : 25% : C20:4 n-6 : 9 % ; DMA : 11%) nor in [PL] repartition (phosphatidylcholine : 70 % ; phosphatidylethanolamine : 25% ; phosphatidylinositol : 4 %) was found after liposom[e] preparation. These results show that no specific loss of peculiar phospholipid class takes place when plugging occurs during LUV extrusi[on] or after SUV centrifugation.

***Characteristics of fresh liposome suspensions*** : Comparison of conjugated dienes and TBA values (fig. 1) and of PUFA content and PL repartion between PL extracts and MLVs showed that no PL alteration was induced by MLV preparation. By contrast, extrusion and sonication induced oxidation as shown by conjugated dienes and TBA values of LUV and SUV. Accordingly, variance analysis perform[ed] on conjugated dienes and TBA values of the two series and the three liposomes types showed that freshly prepared MLVs exhibited lower TBA and conjugated dienes values than LUV and SUV and that SUV had the highest conjugated diene values. Meanwhile, in all cases, these oxidation indices remained very low : for exemple, 30 min after $Fe^{3+}$-ascorbate addition, at 25°C, TBA and diene conjugated values of liposomes were 10 fold increased. Moreover, no change in PUFA content, nor in PL repartition was found upon LUV and SUV making.

As shown in fig. 2, if conjugated dienes values were similar for the two series of preparation, some difference appeared in TBA values.

Turbidity at 350 nm of liposomes was found to be highest for MLV (1.2-1.4), intermediate for LUV (0.24-0.27) and lowest for SUV (0.1[3-] 0.14). This result is due to great differences in size of these liposomes : 500-1000 nm for MLV, 100 nm for LUV and 30 nm for SUV (HUANG, 1969, HOPE *et al*, 1985, LICHTENBERG, 1988 and unpublished results).

e 1 : Influence of the type of liposomes upon oxidation s (conjugated dienes and TBA values) immediatly after mes preparation.

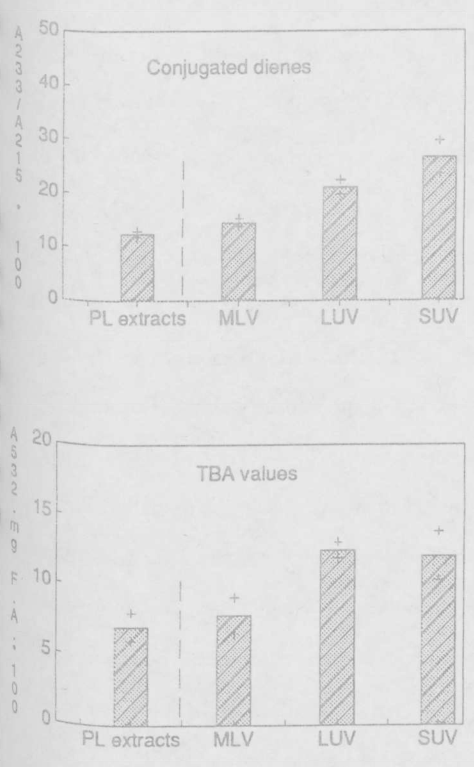
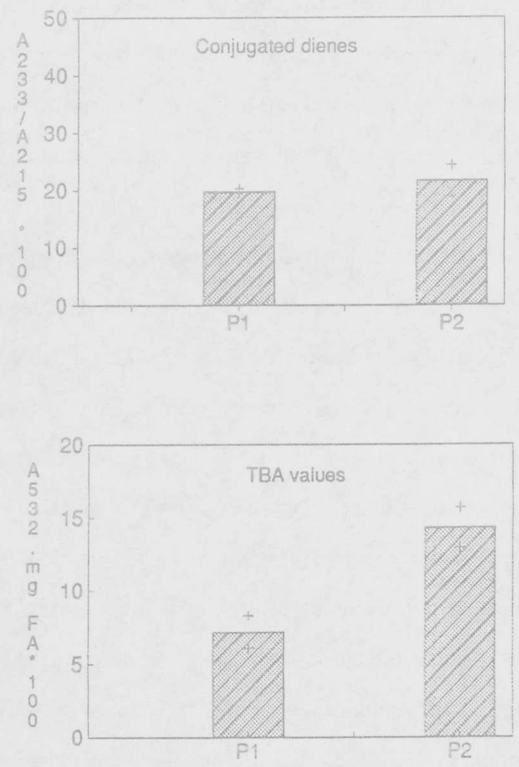
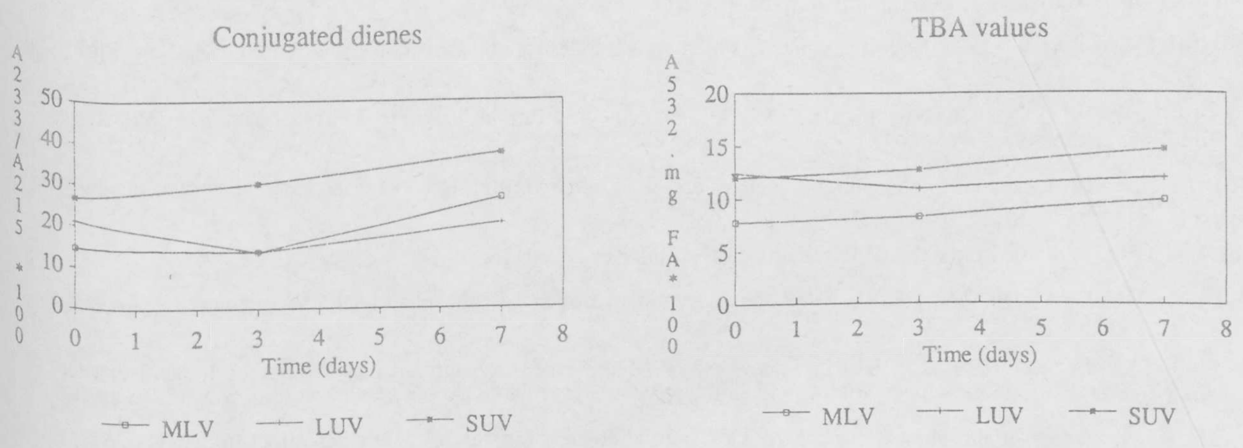



**Figure 2** : Influence of liposomes preparation (P1 and P2) upon conjugated dienes and TBA values of phospholipid liposomes.




*uence of storage* : During storage of liposomes, TBA and conjugated dienes values are constant for 3 days (fig. 3). After 7 days, these es increased for the three types of liposomes. No difference between liposome type was found according to their chemical stability g storage. On the same time no evolution in repartition of PL classes nor in PUFA content of liposomes could be detected during the 7 of storage.

e 3 : Evolution of conjugated dienes and TBA values of liposomes during storage at 4°C in the dark.




idity of MLV was constant over 7 days of storage. This apparent physical stability of MLV should be confirmed using method allowing ecise determination of sizes. For SUV and LUV some increase (SUV : 5-15 % ; LUV : 8-20 %) of the turbidity occured after 3 days of ge, after 14 days of storage, the increase varied between 8 and 30 % for SUV and between 15 and 55% for LUV.

## DISCUSSION and CONCLUSIONS

Some comparison of the methods used to evaluate alterations of PL led to the expected conclusion that conjugated diene measureme the most sensitive method, followed by TBA measurements. These methods are suitable to detect very low oxidation levels, wh determination of PUFA content and determination of PL classes do not allow to detect the early beginning of the oxidation.

In spite of the apparent physical stability of MLV, its easiness of preparation and the good chemical stability of PL during preparati two difficulties remain in the use of MLV in model system : firstly, the great absorbance of MLV ptohibits some spectroscopical metho secondly, due to the broad distribution of vesicle sizes and of number of phospholipid bilayers per vesicle, the analysis of kinetics (as li oxidation kinetics) may become rather difficult.

Liposomes obtained by sonication procedure have two drawbacks too. First, if preparation conditions, as buffer degazing, temperat controlling,.. are not strictly respected, integrity of PL become hazardous. This point is illustrated by the high standard deviation observed TBA and conjugated dienes values of SUV (fig. 2) and agrees with previous works (HO *et al*, 1987, ALMOG *et al*, 1991). Second, SU represents the lowest feasible size for PL vesicles from thermodynamical molecular size and shape considerations. This implies particul constraints and modification in bilayer physical parameter as compared to natural membranes.

Finally, liposomes obtained by the extrusion method (LUV) appear to be the best model to study muscle PL oxidation. When plugging occurs during liposomes extrusion, it is easy to change the polycarbonate membranes with no significant losses in PL. LUV have very lo oxidation levels after preparation. They can be used during 3 days after preparation. Moreover, LUV which are unilamellar, have a ve sharp size distribution, the mean size being determined by the choosen pore size of the polycarbonate membranes. The modifications whic were observed in LUV turbidity within storage can be tentatively attributed to changes in size distribution of vesicles due to aggregatio and/or fusion of vesicles. These results should be confirmed by the mean of methods allowing a precise size determination. Moreover, th influence of liposome structure on catalysed oxidation should be studied now.

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