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Steam sterilisation of vesicular phospholipid gels

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Abstract

Vesicular phospholipid gels (VPGs), highly concentrated phospholipid dispersions of semisolid consistency and vesicular morphology are under investigation as potential implantable depots for sustained release of drugs and as intermediates for subsequent dilution into 'conventional' liposome dispersions. It was investigated here if VPGs can be steam sterilised. VPGs prepared from 400 mg/g egg-phosphatidylcholine by high-pressure homogenisation retained their vesicular structure but showed a slight increase in vesicle size (freeze-fracture electron microscopy). However, autoclaving slowed down both, the in vitro release of the hydrophilic marker carboxyfluorescein and vesicles from VPGs. This was assumed to be due to bigger vesicle sizes and corresponding increase in packing density of the vesicular matrix. Upon dilution into a liposome dispersion both negative staining electron microscopy and dynamic laser light scattering analysis confirmed a distinct increase in liposome size, mainly due to fusion of small (20 nm) vesicles with unfavourable curvature. This was consistent with the observed increase in encapsulation efficiency of carboxyfluorescein. Phospholipid hydrolysis during autoclaving was negligible with lysophosphatidylcholine formation of less than 2% (thin layer chromatography). Despite significant change of their morphological and functional properties during autoclaving VPGs retained their main characteristics, such as vesicular structure, sustained release and dilutability to liposome dispersions, and are, therefore, considered as autoclavable. © 2001 Published by Elsevier Science B.V.

Keywords: Phosphatidylcholine; Liposome; Vesicle; Sterilisation; Autoclaving; Particle size; Hydrolysis

1. Introduction

Vesicular phospholipid gels (VPGs), originally described in Brandl et al. (1994) can be obtained by high-pressure homogenisation of highly concentrated (phospho)-lipid dispersions. They have a semisolid, gel-like consistency and consist of numerous densely packed, small, mostly unilamellar vesicles (SUVs) (Brandl et al., 1997). During in vitro release tests, incorporated hydrophilic markers were found to be released in a sustained

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manner within periods ranging from several hours up to several days depending on the concentration and composition of the (phospho)-lipids within the matrices (Tardi et al., 1998). The markers were released in free as well as in liposome-entrapped form. VPGs could therefore be useful as parenteral depot formulations. Alternatively, by mixing with excess buffer VPGs may be converted to 'conventional' liposome dispersions with small and homogeneous particle sizes but with high encapsulation efficiencies (Brandl et al., 1998). VPGs appear thus useful as intermediates for liposome dispersions, especially those with drugs which have a high leakage rate and poor storage stability such as Gemcitabine (Moog, 1998). In case of parenteral application, VPGs have to comply with the requirements for parenterals, primarily sterility of the preparation. Final steam sterilisation (treating of the finished product in its container in an autoclave) is the method of 1st choice in the European Pharmacopoeia, for aqueous preparations. For liposome dispersions, however, literature reports so far mostly recommend sterile filtration and aseptic filling due to insufficient physical and chemical stability during heating (Amselem et al., 1993; Sorgi and Huang, 1996; Lasic, 1998). New (1990) even considers sterile filtration as the only suitable method for obtaining sterile liposome preparations. Sterile filtration, however, is restricted to liposomes of small sizes (up to approximately 300 nm) and to dispersions of low viscosity. If the liposomes are too big, or as in the case of VPGs the viscosity of the preparation is too high, sterile filtration cannot easily be performed. It can then be considered to carry out the whole production process under aseptic conditions, which entails a high technical and economic expenditure. It would be advantageous thus, if one could use autoclaving as the final sterilisation step. Relatively few reports have been published so far about the steam sterilisation of liposomes (Cherian et al., 1990; Kikuchi et al. 1991; Garelli and Vierling, 1992; Zuidam et al., 1993; Lukyanov and Torchilin, 1994; Choquet et al., 1996). Autoclaving of liposomes is generally regarded problematic because of the risk of physical and chemical degradation of the liposomes and/or of the encapsulated drug. Among these the most serious risk during autoclaving of 'conventional' liposomal dispersions is the loss of encapsulated drug due to leakage (Kikuchi et al. 1991; Zuidam et al., 1993; Choquet et al., 1996).

Alternative methods for the sterilisation of liposomes have been tested: Stricker and coworkers (Mentrup et al., 1989; Stricker et al., 1991) have treated MLV-preparations with high-pressure sterilisation (24 h at 60°C and 250 MPa). Limited experience is available with this method in terms of liposome integrity; its effectiveness in reducing microbial contamination in liposomal preparations still has to be validated. The use of gamma-irradiation for the sterilisation of liposome dispersions has gained interest in the last years (Zuidam et al., 1995; Stensrud, 1999). This technology appears to be of limited use, namely to freeze-dried preparations, as for aqueous dispersions, the hydroxyl radicals (produced by water radiolysis) cause a pronounced chemical degradation of the headgroup of the liposomal phospholipids.

The aim of this study was to investigate the suitability of autoclaving as sterilisation process for vesicular phospholipid gels (VPGs).

2. Materials and methods

2.1. Preparation and autoclaving of vesicular phospholipid gels

Egg-phosphatidylcholine (egg PC) was mixed with phosphate-buffered saline (PBS) or with a 7.25 mM solution of hydrophilic marker (carboxyfluorescein) and allowed to swell for 2 h under magnetic stirring. The raw lipid dispersions containing a total of 40% (m/m) of lipid were homogenised as described earlier ('one-step liposome preparation', (Brandl et al., 1990, 1993)) using a high-pressure homogeniser Micron Lab 40 (APV Gaulin, Lübeck, Germany) at 70 MPa for 10 cycles. An aliquot of the obtained preparation was then autoclaved at 121°C and 2 bar for 15 min according to the standard procedure.

2.2. Electron-microscopical characterisation

Freeze-fracture electron microscopy (FF-TEM) was performed as described previously (Brandl et al., 1997). In brief: small portions of the lipid pastes were mounted on a gold specimen holder (Baltec, Balzers, Liechtenstein) and quick-frozen by plunging into liquid ethane cooled to 77–100 K using liquid nitrogen. After a few seconds the sample was transferred onto a specimen table immersed in liquid nitrogen until insertion into the pre-cooled freeze fracture unit (BAF 301, Baltec, Balzers, Liechtenstein). Fracturing was carried out at 173 K and between 3.5×10^{-6} and 1.4×10^{-5} Pa. The fracture faces were etched for 30 s at 173 K. Subsequently, the etched surface was vacuum deposited unidirectionally with platinum/carbon (ca. 2 nm) and carbon (ca. 30 nm) at an angle of 40–45°. The obtained replica was floated off in ethanol/water or ethanol/chloroform mixtures. Lipid traces were removed by repeated flushing. Visualisation was done on a Philips EM 400 transmission electron microscope operated at 80 kV.

For the negative staining electron microscopic analysis, specimens were prepared according to standard protocols: carbon-coated copper grids (Science Service, D-München) were hydrophilised by glow discharge. A drop of the diluted VPG was placed on the grid, allowed to adsorb, and the excess was removed by blotting paper. A drop of 2% uranyl acetate solution (Serva Feinbiochemica, D-Heidelberg) was added and the surplus was removed again. After drying at ambient conditions, the grids were examined in the electron microscope (Philips EM 400).

2.3. Particle size analysis

Size distributions were determined from negative staining electron microscopic prints at large magnifications (ca. 100 000 ×). For each distribution, the diameters of about 1000 liposomes were measured using a digitising tablet. At the same time, particle size distributions of the liposome dispersions were measured by dynamic laser light scattering using a Malvern Zetamaster (Malvern Instruments, Malvern, Worcestershire, UK).

2.4. In vitro release testing

The release tests were performed using a home-made release testing apparatus based on a flow-through cell as described previously (Tardi et al., 1998). The acceptor compartment of the cell could accommodate about 1 g of the semisolid VPG preparation. The acceptor compartment was continuously rinsed with phosphate buffered saline (PBS, pH 7.4, isotonic) at a flow rate of 10 ml/h. In order to be able to follow the release of liposomes from the VPGs, there was no separating membrane between donor and acceptor compartment. During release tests, the buffer stock and the release cells were kept at 37°C. Fractions were collected over time and each fraction further fractionated by column chromatography into a liposomal and a non-liposomal fraction using Sephadex G 25 medium gel (Pharmacia, Freiburg, Germany). The fluorescence intensity of all collected fractions and of the corresponding subfractions obtained after gel permeation separating free carboxyfluorescein from liposomal carboxyfluorescein, was measured using a Perkin-Elmer LS 50 B luminescence spectrometer (excitation wavelength 492 nm; emission wavelength 514 nm). Any turbidity in the fractions arising from liposomes was removed by addition of excess sodium cholate. The release test was done twice for each preparation.

2.5. Encapsulation efficiency

The gel was first diluted 1:1 with buffer using a ball mill as described in Brandl et al. (1998). It was then fractionated by column chromatography using Sephadex G 25 medium gel (Pharmacia, Freiburg, Germany). The liposomal and free carboxyfluorescein fractions were analysed fluorimetrically as described above. The encapsulation efficiency was calculated as the percentage of liposomal marker compared to total (free and liposomal) marker.

2.6. Hydrolysis

The formation of LPC in the samples was monitored using high performance thin-layer

chromatography (HPTLC). The samples were dissolved in a mixture of *n*-hexane/isopropanol/water (46:46:8 v/v/v) and sprayed onto silica plates using a Desaga AS 30 (Desaga, D-Wiesloch). Plates were developed in chloroform/methanol/water (65:25:4), dipped for 3 s in a 10% copper sulphate in 8% ortho-phosphoric acid solution and heated for 10 min at 170°C in an oven. For quantitative evaluation a densitometer Desaga CD 60 was used. Quantifications were based on reference substance L-α-lysophosphatidylcholine from egg yolk (Sigma, Deisenhofen, Germany).

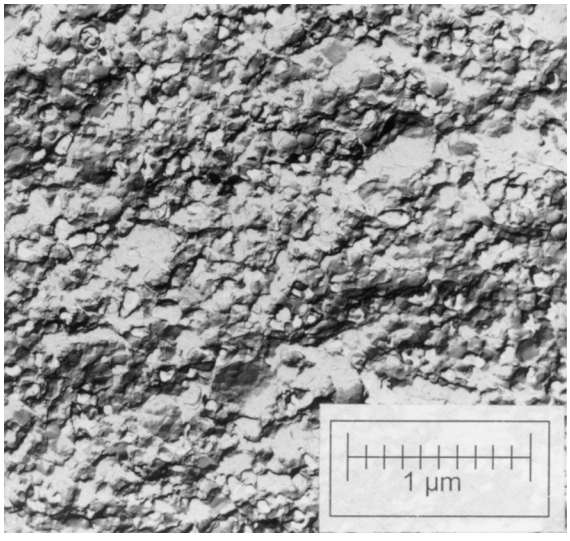

Fig. 1. FF-TEM micrograph of the 40% EPC-gel before autoclaving.

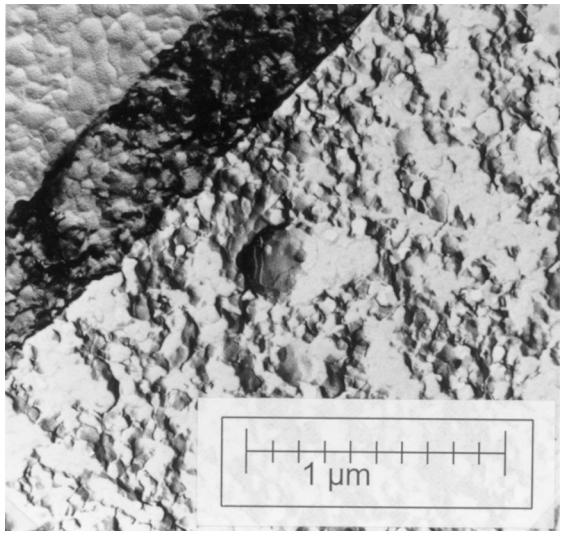

Fig. 2. FF-TEM micrograph of the 40% EPC-gel before autoclaving.

3. Results and discussion

VPGs made of 400 mg/g EPC were prepared and autoclaved at standard conditions, i.e. 15 min at 121°C. The main physical, chemical and biopharmaceutical properties of the gels were assessed before and after autoclaving. The morphology of the gels was studied by means of freeze-fracture electron microscopy. The in vitro release profiles were determined using the low-molecular-mass hydrophilic model substance carboxyfluorescein. The liposomes obtained after re-dispersion of the gels were visualised and sizes determined by negative staining electron microscopy. The resulting liposome size distributions were compared with the results of PCS (photon correlation spectroscopy) measurements. Furthermore, the influence of autoclaving on encapsulation efficiency was studied. The lysophosphatidylcholine (LPC)-content of the gel was analysed in order to assess its chemical stability.

3.1. Morphology of vesicular phospholipid gels

Freeze-fracture electron microscopy allows the visualisation of the morphology of the intact vitrified VPG. Representative FF-TEM micrographs of the VPG before and after autoclaving are shown at different magnifications in Figs. 1–4.

The VPG before autoclaving shows a quite homogeneous morphology with a huge number of round fracture faces, which are assigned to small, mostly unilamellar vesicles. The vast majority of them have a diameter below 100 nm (Fig. 1). At higher magnification (Fig. 2) besides this fracture plane also some surface of the gel can be seen in the left hand upper corner. Here the surface of the vesicles and their round shape can be easily recognised. These observations are in agreement with a previous study of Brandl (Brandl et al., 1997).

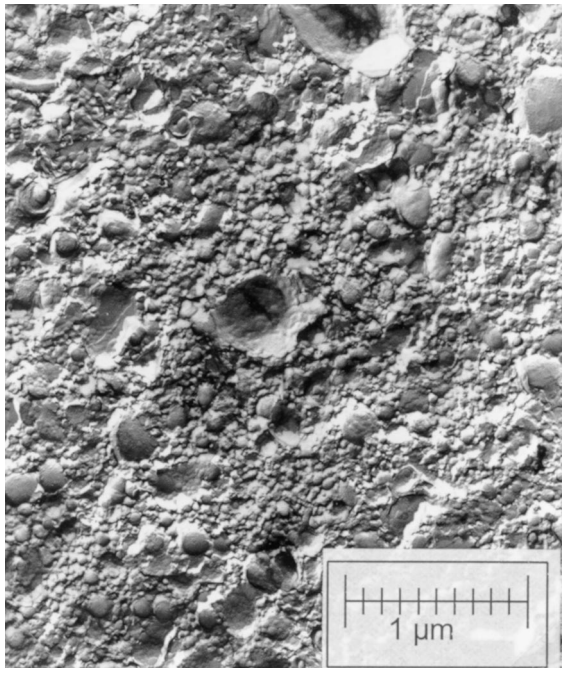

Fig. 3. FF-TEM micrograph of the 40% EPC-gel after autoclaving.

After autoclaving, a slightly different picture is obtained (Fig. 3): still, the VPG is composed of tightly packed vesicles, and, still most of them are very small, below 100 nm in diameter. Some of them, however, have a size between 100 nm to 500 nm. It appears as if these bigger vesicles are more numerous as in the gel before autoclaving but it is difficult to derive reliable particle size distributions from freeze-fracture micrographs because the vesicles may be fractured not equatorially and the fracturing behaviour of smaller and bigger vesicles may differ in this respect. At a higher magnification (Fig. 4) the vesicular structures can be easily recognised in more detail: the bigger vesicles seem to represent partly multilamellar vesicles and partly multivesicular vesicles, i.e. vesicles with a surrounding membrane and an interior consisiting of tightly packed smaller vesicles. The occurrence of such multivesicular vesicles in VPGs has earlier been described (Brandl et al., 1997).

In conclusion: the vesicular structure is maintained after autoclaving. However, bigger vesicles appear to be more numerous. As only a small fraction of the sample is being observed, it might not be representative for the whole preparation. It cannot definitely be concluded from these micrographs that autoclaving induces a growth of the vesicles, and if, to which extent. On the other hand, vesicle size is regarded to be of minor importance if one only intends to apply the VPG intramuscularly or subcutaneously. Of much greater importance is if the functionality of the VPG is affected by autoclaving.

3.2. Sustained release

Figs. 9 and 10 show the in-vitro release behaviour of a 400 mg/g EPC-gel before and after autoclaving, respectively. The release of the marker carboxyfluorescein, both in free and in liposomal form and the overall release was measured over time. The total period over which the release occurred was found extended from 7 to 28 h after autoclaving, i.e. the CF release from the autoclaved gel was four times slower. This repre-

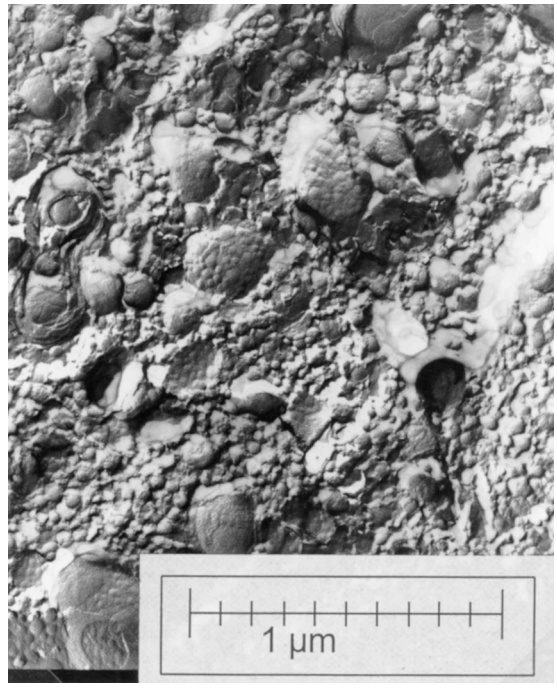

Fig. 4. FF-TEM micrograph of the 40% EPC-gel after autoclaving.

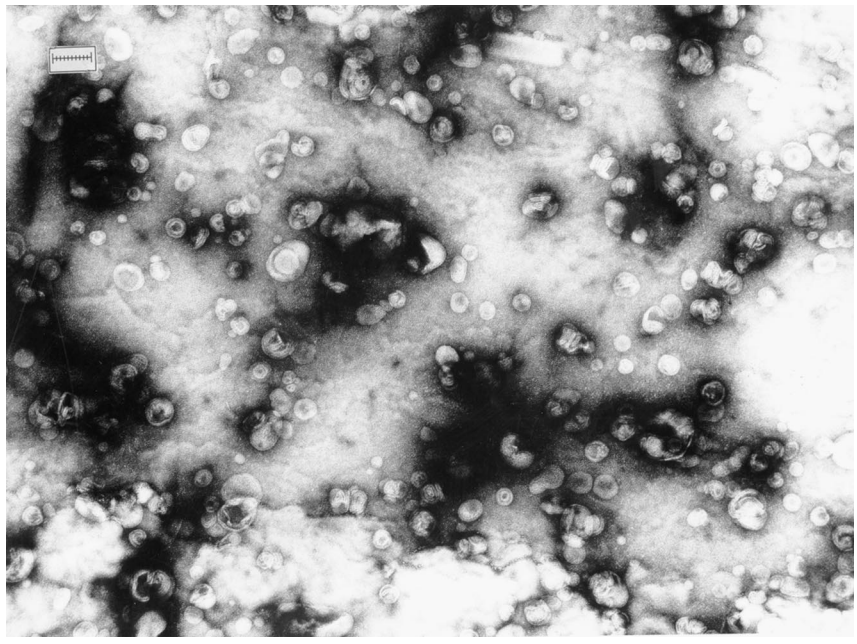

Fig. 5. NS-EM micrograph of the non-autoclaved gel after redispersion. Bar is 100 nm.

sents a strong change of the function of the gel. Nevertheless, the proportion of CF released in liposomal form as compared to totally released CF stayed nearly the same. Moreover, the CF-release in liposomal form followed in both cases a zero order kinetic, which is taken as a hint for a constant erosion process. Taking into account the above results concerning liposome size it may be concluded that the increase of liposome size during autoclaving might be responsible for the slowing down of the release process. Theoretical considerations on the packing density of the vesicles may explain this: At constant phospholipid content a smaller number of bigger vesicles takes more space than a higher number of smaller vesicles. Despite the tighter packing, release of the CF in liposomal form seems to occur at a constant but slower rate.

In conclusion firstly autoclaving caused a drastic alteration of the release behaviour of the VPG studied. Such alteration must be taken into account when designing future VPG systems. Secondly it should be studied if and how autoclaving affects the characteristics of liposome dispersions obtained by dilution of VPGs. Especially if redispersed VPGs are intended for i.v. administration vesicle size plays a key role.

3.3. Vesicle size distribution of redispersed VPGs

The gels were redispersed with buffer using a ball mill and the resulting liposome dispersions which contained about 1% (m/m) of total lipid were analysed by NS-EM. This method allows the visualisation of liposomes as separate, single vesicles. Figs. 5 and 6 show typical NS-EM micrographs of the redispersed VPG, both, before and after autoclaving, respectively. The vesicles were in both cases small, with a diameters mostly between 20 and 100 nm. In the autoclaved preparation, a few bigger liposomes with a size up to 250 nm could be seen. These observations are in agreement with those made using FF-TEM micrographs of the non diluted VPG.

The diameters of about 1000 vesicles per preparation were determined from the micrographs using a digitising tablet. The resulting size distributions for both the autoclaved and non-autoclaved VPG are shown in Fig. 7. Both distributions are typical in shape for liposome dispersions

prepared by high-pressure homogenisation: they are quite narrow and asymmetrical, i.e. steep to the left, as described previously (Brandl et al., 1990, 1993). After autoclaving a decrease in the fraction of the smallest vesicles (below 20 nm) is observed. This is in good agreement with theoret-

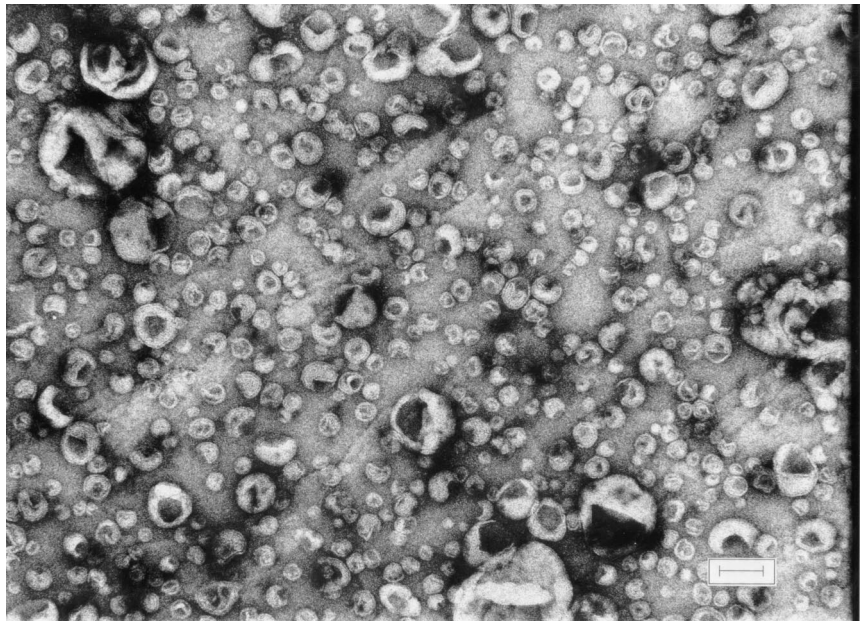

Fig. 6. NS-EM micrograph of the autoclaved gel after redispersion. Bar is 100 nm.

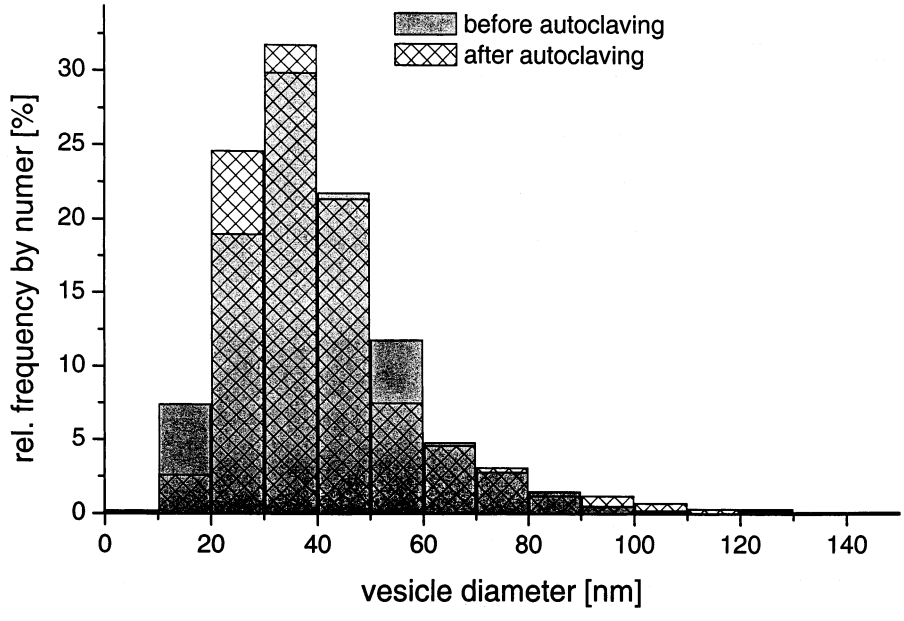

Fig. 7. Size distribution of the redispersed gels, number based histogram. Dark bars: before autoclaving; cross hatched bars: after autoclaving.

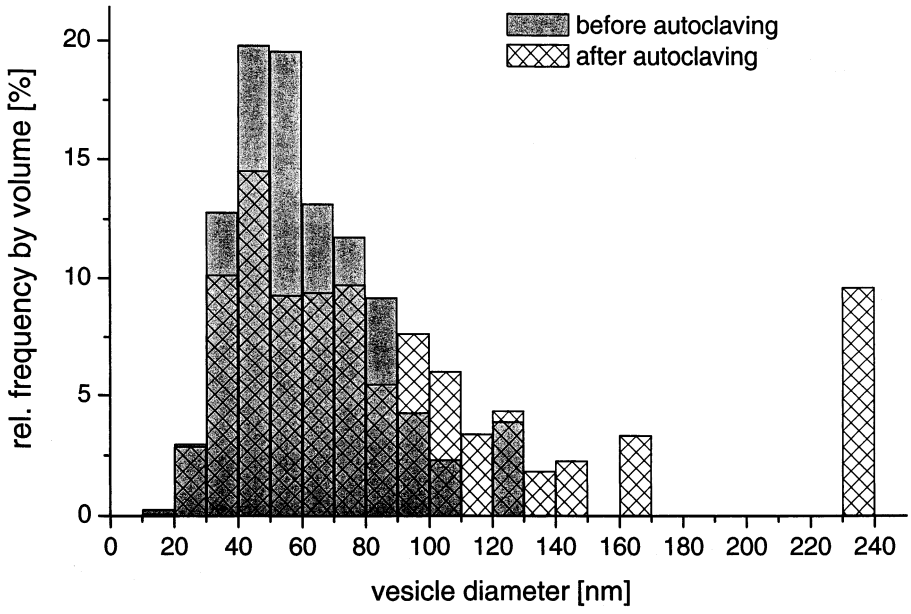

Fig. 8. Size distribution per volume of the redispersed gels, volume-based histogram. Dark bars: before autoclaving; cross hatched bars: after autoclaving.

ical expectations that vesicles smaller than 20 nm are very unstable because of their high degree of curvature (Brandl et al., 1993). At the same time, a slight increase of the number of vesicles with diameters of 50 nm and above could be observed. Apparently a fusion process occurred during autoclaving. For both, the autoclaved and the non-autoclaved preparation, more than 90% of the vesicles were observed in the size range between 20 and 80 nm and more than 70% of the vesicles were counted between 20 nm and 50 nm. No major difference could be derived between the gels from the comparison of their size distribution by number except the disappearance of vesicles below 20 nm in diameter during autoclaving.

On the contrary, the PCS results clearly indicated a strong growth of the mean vesicle size after autoclaving and redispersion: the Z-average increased from 98.7 ± 2.5 to 154.6 ± 1.6 nm and the modus (peak) of the size distribution (by number) was found raised from 62.1 to 94.1 nm after autoclaving.

It was assumed that this discrepancy between the particle size distributions gained by NS-EM and PCS, respectively, might be due to the different basis of the distributions (number-based versus intensity/mass/volume-based) and/or due to the much higher sensitivity of the light scattering technique towards bigger particles. The number-based vesicle size distributions gained by negative staining electron microscopy were thus, under the assumption that liposomes represent spheres, mathematically converted into volume-based vesicle size distributions (Fig. 8). This led to a quite different impression: vesicles in the range up to 100 nm represented before autoclaving more than 95% of the total vesicle volume, whereas after autoclaving only 75% of the total volume. Although there was less than one out of a hundred vesicles found with a diameter of more than 100 nm, this size fraction represented 25% of the total vesicle volume after autoclaving. No vesicles bigger than 300 nm were observed. It has to be mentioned, however, that it is difficult to detect such big vesicles by NS-EM, because they cannot be clearly distinguished from artefacts. The NS-EM analysis of the vesicle size distributions by volume and PCS measurements thus congruently indicated a growth of the vesicle sizes during autoclaving.

These results differ from those reported in the literature, where the liposomes were found to maintain their original sizes (Zuidam et al., 1993; Lukyanov and Torchilin, 1994; Choquet et al.,

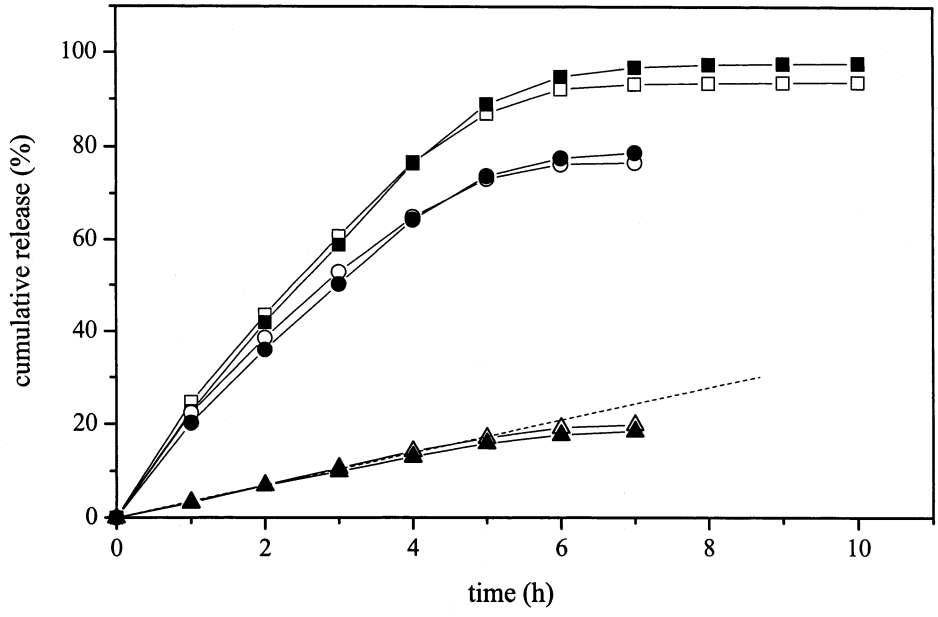

Fig. 9. CF-release from a 40% EPC-gel. —□— , Total; —○—, free; —△— liposomal. Open symbols, first run; solid symbols, second run.----, Linear fit of the liposomal CF release over the time period 0–5 h, $r = 0.9994$.

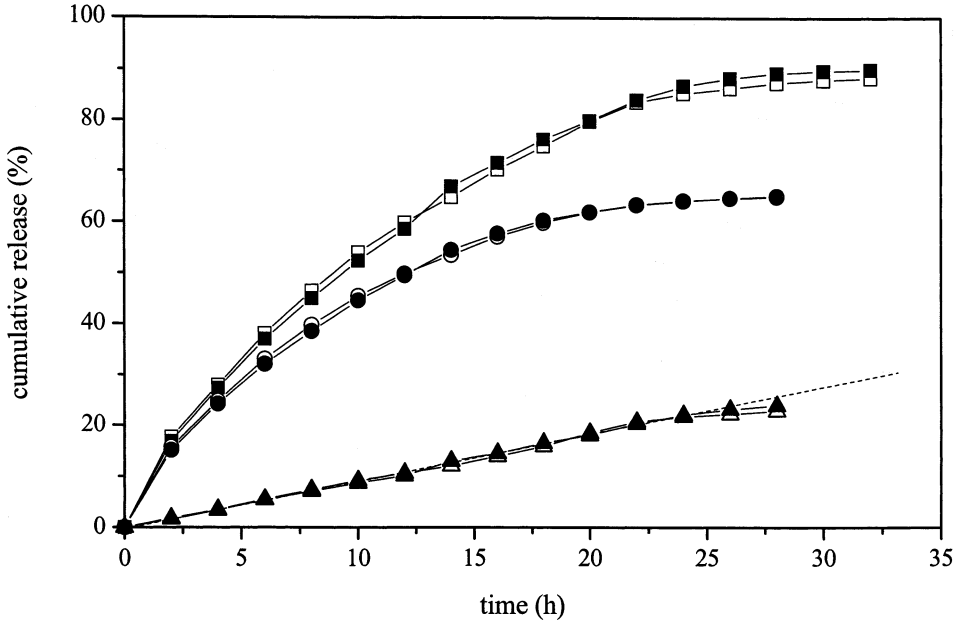

Fig. 10. CF-release from an autoclaved 40% EPC-gel. —□— , Total; —○—, free; —△— liposomal. Open symbols, first run; solid symbols, second run.----, Linear fit of the liposomal CF release over the time period 0–24 h, $r = 0.9993$.

1996). Other than here, these studies were made using 'conventional' liposome dispersions of much lower lipid content (dispersions with ca. 30 mM lipid) and relatively big liposomes (approximately 100 nm in diameter). It can be assumed that these liposomes do not have such a curvature and thus high fusion tendency as the very small high-pressure homogenised liposomes. When looking at an intravenous application of redispersed VPGs it has to be mentioned, however, that the vesicle sizes observed after autoclaving and redispersion are still relatively small compared to other liposome dispersions intended for i.v. application in the literature (Amselem et al., 1993).

3.4. Encapsulation efficiency (EE)

First measurements were performed using calcein, a fluorescent molecule, as a hydrophilic model substance but it was found to be unstable upon autoclaving. The autoclaved calcein was found unable to pass through a Sephadex gel filtration column and had much lower fluorescence intensity. As no such changes were observed with carboxyfluorescein, the latter was used for further experiments.

After autoclaving the encapsulation efficiency of the carboxyfluorescein-containing VPG was found increased from 29.5% ($n=2$) to $47.0 \pm 0.9\%$ ($n=3$). This strong increase of the encapsulation efficiency is in good agreement with the observed growth of the liposome size if one takes into account that non-entrapped carboxyfluorescein is present during autoclaving. The very small liposomes below 20 nm with unfavourable volume to surface ratio fuse to bigger vesicles which leads to an overall increase in entrapped volume. One of the main problems occurring during autoclaving of conventional liposome dispersions is the leakage of the encapsulated drug out of the vesicles. With the VPG, a very high proportion of the drug is encapsulated in the liposomes. Therefore, a removal of the non-encapsulated drug is in many cases not necessary. As a consequence, no concentration gradient occurs between the intra- and the intervesicular space, and the increased membrane permeability during autoclaving does not lead to a significant net drug flow across the membrane. The encapsulation efficiency is maintained or it increases proportionally to the vesicle size. In this respect, the VPG concept allows a clear improvement over conventional liposomes when it comes to stability during steam sterilisation.

3.5. Hydrolysis

Phospholipid can undergo hydrolysis of its ester links between the glycerol backbone and the fatty acids, preferably on *sn*-2. By this way the intermediate degradation product lyso-PC is formed, which subsequently may degrade to glycerophosphocholine and a second fatty acid. As the first hydrolysis step is faster, an accumulation of lyso-PC usually is observed, which may serve as an indicator for the overall degradation process. At the beginning of the hydrolysis process, the decrease of phosphatidylcholine content and formation of glycerophosphocholine cannot be measured with sufficient precision. Lyso-PC was thus quantified here to follow the hydrolysis of PC, although it represents an unstable intermediate and may thus lead to an underestimation of the hydrolysis reaction rate.

LPC-molecules themselves cannot form vesicles but they have strong surfactant properties. At low concentrations they can be incorporated into the liposome membranes, whereas at high concentrations, they form mixed micelles with phospholipids, thus causing lysis of the liposomes (Israelachvili, 1985). An increased LPC-content in EPC-liposomes can also induce toxic effects upon i.v. administration (Lutz et al., 1995). It is therefore important to check that the LPC-content does not reach a value where it might affect the integrity of the liposomes and/or be potentially toxic.

No LPC could be found in the raw material (EPC of the same batch as the one used to prepare the VPG). The detection limit was approximately 0.1% LPC, related to the phospholipid content. In the autoclaved gel, 1.59% LPC were found, related to the phospholipid content. This content is rather low. It corresponds to 2.5 mol.%. According to Lasic (Lasic, 1993), small

quantities of LPC (up to 7 mol.%) have a stabilising effect on liposomes, due to their preferred localisation in the outer membrane of the liposomes. The preparation can therefore be considered to be stable upon autoclaving with respect to hydrolysis. This is in agreement with the results of Zuidam (Zuidam et al., 1993) who found during autoclaving of liposomes at physiological pH less than 1.2% hydrolysis of the phospholipids.

In summary: Autoclaving of VPGs caused a fusion of primarily small vesicles of unfavourable curvature, which in consequence resulted in a higher packing density and thus slower erosion of the vesicular matrix during in-vitro release tests. This, in turn, led to a dramatically slower release of the marker. It remains to be studied if this change in functionality during autoclaving can be reproduced well enough to take it into account when designing future VPG-based sustained release systems. For VPGs to be redispersed into 'conventional' liposome dispersions for i.v. application the observed shift towards bigger vesicle sizes appears irrelevant for safety but might well affect the pharmacokinetic behaviour of the lipososomes. VPGs are in principle considered as autoclavable. Autoclaved VPGs have already been used in first animal experiments (Moog, 1998).

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