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European Journal of Pharmaceutics and Biopharmaceutics 54 (2002) 83–87

European Journal of Pharmaceutics and Biopharmaceutics

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Research paper

# Long term stability of rh-Cu/Zn-superoxide dismutase (SOD)-liposomes prepared by the cross-flow injection technique following International Conference on Harmonisation (ICH)-guidelines

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Received 17 July 2001; accepted in revised form 18 April 2002

**Abstract**

The current market position of liposomes as drug carriers is still being discussed with regard to large scale production, product characterisation and the stability of the dispersions. In this study, long term stability of liposomal suspensions with encapsulated rh-Cu/Zn-superoxide dismutase was tested according to the International Conference on Harmonisation (ICH) recommendations. The guidelines of the ICH provide general requirements for stability testing for registration and export in particular. The Institute of Applied Microbiology has examined a process to produce large amounts of pharmaceutical-grade liposomes for the treatment of inflammatory diseases by topical application. For the evaluation of its long-term storage stability, liposomal stability and protein stability were tested under appropriate conditions. Therefore, size alterations of the vesicles, protein release and protein activity were evaluated. During the observation period, neither significant alterations of the liposomes nor any protein degradation could be detected. In the light of these findings our liposomal formulations seem to provide chemical, physical and biological stability according to the definitions of the ICH. Appropriate lipid compounds and environmental factors, in combination with an optimised process and adequate storage conditions, facilitate the production of liposome dispersions suitable as drug carriers on the market. 

*Keywords*: Liposomes; Superoxide dismutase (rh-Cu/Zn SOD); Stability; International Conference on Harmonisation

## 1. Introduction

Currently, the possibility of using liposomes as drug carriers is being discussed under the aspects of large scale production, product characterisation and stability of the dispersions. As mentioned in the International Conference on Harmonisation (ICH) guidelines, the stability of new products, in the present case, a biotechnologically derived protein encapsulated into liposomes, have to meet the requirements of biotechnological products. The ICH guidelines provide a general proposal on the requirements for stability testing, but leave sufficient flexibility to encompass the variety of different practical situations [1–3]. The purpose of stability testing is to prove how the quality of a drug varies with time under the influence of a variety of environmental factors. Stability information from long term testing should cover a minimum period of 12 months. Testing should also cover those features susceptible to change during storage and likely to influence the quality, safety and/or efficacy. Therefore, physical, chemical and microbiological test characteristics should be evaluated by validated methods, suitable to detect the changes during storage.

The liposomal product which we manufacture consists of a biotechnologically derived protein, the recombinant human Cu/Zn-superoxide dismutase (rh-Cu/Zn-SOD) expressed in *Escherichia coli*, encapsulated into liposomes [4,5]. To encapsulate this protein into liposomes should first prolong its biological half life and second make the product suitable for a non-invasive topical application. Based on this concept, patients suffering from Peyronie's disease – a painful disease characterised by a variety of symptoms which is strongly related with inflammatory processes initiated by oxygen free radicals – were treated with liposomally encapsulated SOD. The proof of this concept was so encouraging that further clinical studies were performed [6].

The production was performed by a novel cross-flow injection technique, developed at the Institute of Applied

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0939-6411/02/$ - see front matter 
PII: S0939-6411(02)00045-0

Microbiology in co-operation with Polymun Scientific. Cross-flow injection is suitable to produce large amounts of vesicles under mild and sterile conditions whereby non-encapsulated protein and excess ethanol were removed by continuous ultra/dia-filtration. The final product was characterised by its particle size, its distribution, the encapsulated and free protein content and the protein activity. Additionally, all batches were microbiologically tested [7].

Prior to encapsulation, the protein solution itself was tested for long-term stability. Stability could be maintained for at least 3 years' duration.

In particular, the testing of the liposomal products is even more complex, in terms of chemical and physical stability of the vesicles, protein release and protein activity [8]. The liposomal stability was tested by measuring size and size distribution [9]. All analytical procedures applied in stability testing were evaluated in accordance with the requirements of the ICH guideline.

The samples for stability testing were stored at 5 ± 3°C and only sterile product was tested. In terms of storage conditions, according to the ICH recommendations, different temperatures were evaluated.

## 2. Materials and methods

### 2.1. Materials

Dipalmitoyl-phosphatidyl-cholin (DPPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Stearylamine was purchased from Sigma (St. Louis, MO). DPPC, cholesterol and stearylamine were used for vesicle preparation in a molar ratio of 7:2:1. Phosphate buffered saline (PBS, pH = 7.2–7.4) was used as a hydration buffer.

As substance to be incorporated, recombinant human superoxide dismutase (rh-Cu/Zn-SOD) expressed in *E. coli* was used. This therapeutically interesting protein is available at the Institute of Applied Microbiology (IAM), produced in clinical-grade quality within the project sponsored by POLYMUN Scientific GmbH. The protein, rh-Cu/Zn-SOD, contains one Cu and Zn per subunit and forms a dimer with a molecular weight of about 33 kDa. Its biological function is the dismutation of oxygen radicals, which are in turn responsible for painful tissue damage during inflammatory processes.

### 2.2. Liposome preparation

Liposomes were produced by the cross-flow triple injection technique. Cross-flow injection is an advancement of the ethanol injection technique, first described by Batzri and Korn [10]. The production plant is designed to prepare sterile liposome suspensions without demanding further final product sterilisation. Therefore, all parts of the equipment are qualified for depyrogenisation and heat sterilisation. All reagents, such as the buffer solution, the protein solution and the lipid-ethanol solution were transferred into the containments by filtration through 0.22 μm filters. Nitrogen for the injection process was also filtered through a 0.22 μm filtration unit.

Lipid vesicles are formed in the cross-flow injection module at 55 ± 2°C by means of injecting lipids solubilised in ethanol into protein containing buffer. The cross-flow injection module used for liposome manufacture is made of two stainless steel tubes welded together to form a cross. At the connecting point the module has an injection hole drilled by spark erosion (250 μm drill hole).

Immediately after the lipids are distributed into the aqueous protein solution, there are formed so-called planar bilayer fragments which reassemble to liposomes. To obtain reproducible batch profiles, both solutions are continuously transferred through the module in a controlled manner.

#### 2.2.1. Filtration

Non-entrapped protein was separated by an ultra/dia-filtration equipment (Sartocon Slice cassettes, Polysulfone, cut off: 100 kDa). This module provides excellent chemical and thermo stability, thus sterile filtration conditions are guaranteed.

### 2.3. Analytical methods

All analytical methods are qualified and validated for the intended purpose.

#### 2.3.1. Liposome size distribution

Liposome size and size distribution were measured by a novel technique using flow cytometry [9]. The size determination was performed using a FACS Vantage (Becton Dickinson, San Jose, CA), equipped with a 5W Argon Laser (Coherent Innova 305, St. Clara, CA). The laser was tuned to 488 nm and an output of 500 mW. Fl1 at linear amplification was used as threshold parameter (700V, Gain 16). Forward scatter height (FSC-H, scattering angle 10°) and side scatter height (SSC-H, scattering angle 90°) were set to logarithmic amplification to accommodate the entire size range of 26 nm to 1 μm. In each sample, 10 000 particles were measured.

#### 2.3.2. Quantification of entrapped and non-entrapped drug

The amount of entrapped and non-entrapped protein was determined by an enzyme-linked-immuno-sorbent-assay (ELISA) established at the IAM.

In order to determine the internal protein content, the liposome sample is first separated from non-entrapped protein by filtration. In a next step, the sample is diluted with a detergent and then incubated at 37 ± 2°C for at least 120 min. This is necessary to disintegrate the lipid bilayers and leads to micelle formation of the membrane compounds so that the encapsulated protein is released [11]. The non-entrapped protein is quantified in the filtrate. The filtration procedure is validated as well.

The relevant rh-Cu/Zn-SOD samples are tested in a two-

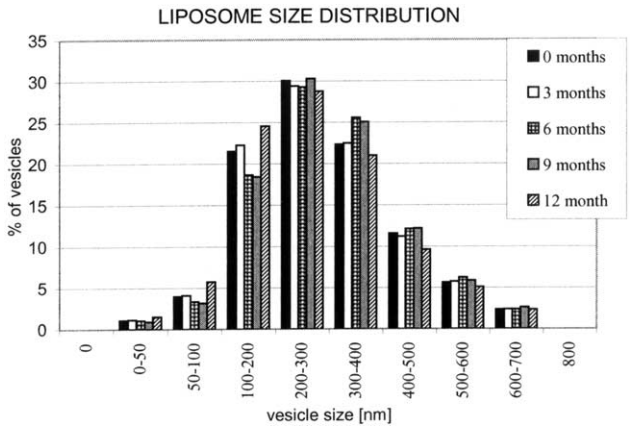


Fig. 1. Shows liposome distributions of one dispersion stored at 5 ± 3°C. This distribution was measured by flow cytometry. In each individual size class the percentage of a total of 10 000 particles are calculated and illustrated in this graph.

step ELISA, in which the immobilised capture antibody is different from the alkaline phosphatase conjugated labelling antibody. After staining, the plates are measured at 405 nm with a reference wavelength of 690 nm. The quantity of incorporated protein can be calculated by two different methods: one is to estimate the total rh-Cu/Zn-SOD content of the sample minus the filtrate content and the other is to measure the retentate protein amount. Both methods yielded similar results for the entrapped enzyme.

### 2.3.3. Protein activity assay

This microtiter assay is based on the enzymatic oxidation of xanthin by xanthinoxidase leading to the generation of free oxygen radicals. The active superoxide dismutase converts these free radicals to $H_2O_2$. Admitted catalase removes the created $H_2O_2$. The radicals reduce nitrobluetetrazolium to its formazan, which can be quantified by photometric analysis at 590 nm. The sample preparation is similar to the ELISA.

Table 1
The aliquotes of the liposome dispersion, stored at 5 ± 3°C, were tested quarterly[a]

| Date of analysis (month) | Entrapped protein concentration (mg/ml ± SD in mg) | Entrapped protein activity (mg/ml ± SD in mg) | Protein release (%) |
|---|---|---|---|
| 0 | 3.14 ± 0.1 | 3.27 ± 0.2 | 0.95 |
| 3 | 3.42 ± 0.2 | 3.00 ± 0.2 | 1.25 |
| 6 | 3.15 ± 0.1 | 3.49 ± 0.2 | 1.30 |
| 9 | 3.10 ± 0.1 | 3.14 ± 0.1 | 1.25 |
| 12 | 3.58 ± 0.2 | 3.68 ± 0.2 | 1.10 |

[a] The drug release is calculated from the ratio of non-entrapped to entrapped protein in percent based on the mean values determined in triplicates.

### 2.3.4. Microbiological testing

Microbiological testing for the absence of colony-forming units was performed in accordance with the European Pharmacopoeia guidelines laid out in chapters 2.6.12 and 2.6.13. As only sterile liposome suspensions are qualified for stability testing, aliquotes were taken into appropriate containers under sterile conditions.

### 2.3.5. Determination of ethanol

Liposomes were separated and clear solutions were analysed by an ion-exclusion high performance liquid chromatography (HPLC) method. The HPLC apparatus was a Beckman System Gold equipped with a refractive index detector (San Ramon, CA). The Organic Acid Column, Aminex Ion Exclusion HPX-87H (Biorad, Richmond, CA), 300 × 7.8 mm was operated at 20 ± 2°C with 100% HPLC grade 0.01 N sulfuric acid at a flow rate of 0.45 ml/min. The injection volume was 20 μl. Ethanol was detected by the refractive index at 30 ± 2°C.

## 3. Results

The chemical and physical stability of lipid vesicles is closely related to their bilayer structures, thus their alterations are detectable in changes of the particle size and size distribution. Therefore, samples of the final liposome dispersions were tested periodically within 12 months (Fig. 1).

Both the entrapped and released protein contents were measured in parallel. The evaluation was performed as described in materials and methods. In addition to the drug release, the loss of activity was evaluated (Table 1).

Furthermore, the influence of temperature was evaluated. In general, all liposomal preparations were stored at 5 ± 3°C. For stability testing additional samples were stored at higher temperatures for at least 3 days, up to the transition temperature (47.8 ± 0.3°C) of the lipid mixture. Those experiments were performed in line with ICH recommendations to prove the influence of temperature fluctuations during transport. As shown in Fig. 2, no alterations of the vesicles were observed. Their size distribution remained constant, even when the temperature increased up to the transition temperature of the lipid mixture.

In parallel, the protein release was evaluated. Results showed only a slight release of the encapsulated material when temperature increased, as shown in Table 2.

## 4. Discussion

The pharmacokinetic behaviour of liposomal associated drugs depends on a number of physical parameters. The chemical and physical stability of liposomes is closely related to the packing density of the bilayers which reduce the influx of oxidising and hydrolysing agents. Therefore decreased alteration in size, fusion properties and mechan-

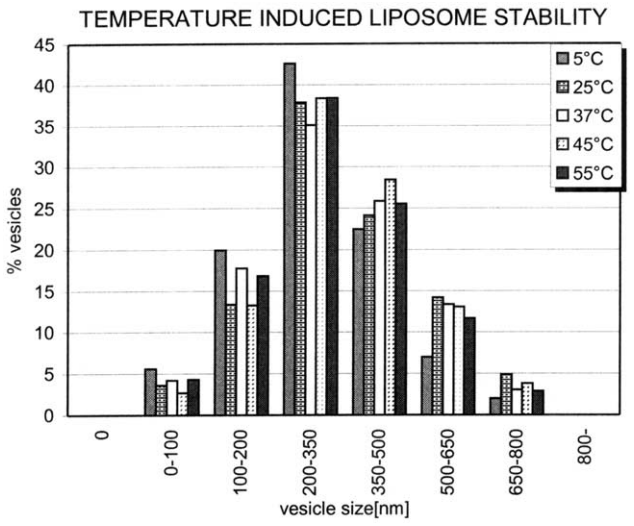


Fig. 2. Shows the effect of several storage temperatures on the vesicle distribution. The distribution with increased temperature differs only slightly from the distribution under standard storage conditions at 5 ± 3°C.

ical properties of the lipid bilayer are expected [12]. The characteristics of the membrane components are an integral part of the stability of the vesicles. Compounds with long and saturated alkyl chains, such as DPPC, provide rigid bilayers with low permeability [13]. Additionally, cholesterol is added to further stabilise the membrane and reduce drug release [14]. Furthermore, our preparation method is designed to remove excess ethanol by continuous ultra/diafiltration in order to reduce destabilising potential. This was done because otherwise the use of large amounts of cholesterol may become problematic, for instance in the presence of larger amounts of ethanol in the dispersion [15]. In the final product, the adopted ethanol concentration amounts to 1%. The use of charged compounds, for instance stearylamine, further decreases potential aggregation and eventual fusion of the vesicles. But not only do the compounds themselves influence the vesicle stability, also the probability of aggregation and/or fusion is strongly dependent on pH, transition temperature and the buffer system [16]. The chemical degradation process during storage may occur upon hydrolysis and oxidation [17]. Oxidation can be neglected in the present context because only saturated compounds are used, and preparation is performed in a nitrogen atmosphere. Otherwise, hydrolysis may be a more serious aspect which may result in glycero-phospho compounds. Hydrolysis is catalysed by protons and hydroxyl ions reaches a minimum at neutral pH. This is one reason why we use PBS (pH = 7.2–7.4) as a hydration buffer. The other reason for using this buffer is the compatibility of this buffer with the protein. Our results, in regard to the particle size and the distribution demonstrate that the choice of robust components in combination with appropriate chemical environments and mild preparation procedures increase the probability of producing stable vesicles.

Table 2
The amount of non-entrapped and entrapped protein was measured by the ELISA[a]

| Temperature (°C ± 3) | Entrapped protein concentration (mg/ml) | Protein release (%) |
|---|---|---|
| 5 | 3.14 ± 0.1 | 0.50 |
| 25 | 3.20 ± 0.1 | 0.64 |
| 37 | 3.38 ± 0.2 | 0.65 |
| 45 | 3.09 ± 0.1 | 0.78 |
| 55 | 3.26 ± 0.2 | 1.16 |

[a] The drug release is calculated from the ratio of non-entrapped to entrapped protein in percent based on the mean values determined in triplicates.

The stability of liposomes, in particular, is reflected in maintaining the encapsulated protein content. To ensure that released protein does not interact with the membranes and therefore is not detectable, studies about protein interaction with liposomes, examined by differential scanning calorimetry (DSC) were performed. In 1995 Yi-Li-Lo and Yueh-Erh-Rahman [18] published a study, using SOD as model molecule, representing a hydrophilic protein. The authors established a highly sensitive DSC to study protein interactions with different liposomes. DPPC vesicles combined with increasing SOD concentration were measured to estimate the $T_m$ (temperature where the transition is half completed), $\Delta T_{1/2}$ (sharpness of the phase transition), $\Delta H$ (transition enthalpy) and CUS (ratio of the Van't Hoff enthalpy and the calorimetric enthalpy). No differences were detectable between pure liposomes and SOD containing suspensions. We performed comparable measurements with our DPPC-liposomes with the same results, thus no interactions between the protein and the liposomes is expected.

During the observation period no detectable drug release was measured if the product was stored at 5 ± 3°C. Additionally, stability data of samples stored at different temperatures show only a marginal protein release, even though they were stored at higher temperatures, for instance near the transition temperature, which is not recommended [8]. In general, liposomes which have a gel-to-liquid phase transition, release encapsulated drug more frequently at higher temperature. However, not only the protein release is important with respect to stability testing. The activity of the rh-Cu/Zn-SOD is just as important. It is difficult to administer both therapeutically inactive material and degradation products, which are considered to be impurities and may induce unwanted side effects. According to the ICH guidelines concerning stability testing of biotechnological/biological products, it is demanded to ensure maintenance of biological activity and to avoid degradation. For the products, in which the active components are typically proteins and/or peptides, maintenance of molecular conformation and, hence of biological activity, is dependent on non-covalent as well as covalent forces. The product is particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and

shear. In the light of these findings, our liposomal dispersions seem to remain fully active rh-Cu/Zn-SOD, implicating stable conformation of the protein.

In conclusion, our data suggest that the stability testing adhering to the ICH guidelines is strongly related with the product, the production process and the storage conditions and should be followed when pharmaceutical, respectively clinical grade material has to be produced.

## Acknowledgements

These studies were supported by Polymun Scientific, Immunbiologische Forschungs GmbH, Nussdorfer Laende 11, A-1190 Vienna, Austria and the Austrian Industrial Research Promotion Fund (FFF) Project Nr.: 804190.

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