ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 54 (2002) 213–219

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Liposomes produced in a pilot scale: production, purification and efficiency aspects

Andreas Wagner[a,*], Karola Vorauer-Uhl[b], Hermann Katinger[b]

[a]*Polymun Scientific, Immunbiologische Forschung GmbH, Vienna, Austria*
[b]*Institute of Applied Microbiology, University of Agricultural Sciences, Vienna, Austria*

Received 21 January 2002; accepted in revised form 26 April 2002

Abstract

Intensive efforts have been made to establish a novel, scalable liposomal preparation technique suitable for the entrapment of even large proteins into liposomes. We have developed a new technique based on the principles of the ethanol injection technique. Herein, the principal item is the crossflow injection module, specifically designed for this purpose. This unit has the benefit of defined and characterized injection streams and permits liposome manufacture regardless of production scale, as scale is determined only by the free disposable vessel volumes. Previous publications demonstrated that the crossflow injection technique that we have developed meets all of the above-mentioned requirements. The present paper describes the entire three-step production process, consisting of encapsulation, separation of non-entrapped protein by continuous crossflow filtration, and retrieval of rh-Cu/Zn-SOD by additional filtration. Results of consecutive lots were compared, based on well-defined quality criteria.

Keywords: Liposome production; Superoxide dismutase; Clinical studies; Pilot scale; Liposome filtration

1. Introduction

Over the past few years liposomal drug preparations have been increasingly used in clinical trials [1–4]. These clinical trials have guided liposomes from laboratory research to clinical reality with very encouraging results.

Searching for new formulations of therapeutically interesting substances, which are produced in fermentation processes by the project sponsor Polymun Scientific and the Institute of Applied Microbiology (IAM), we established several published liposome preparation techniques. One of the therapeutic substances was recombinant human superoxide dismutase (rh-Cu/Zn-SOD), which has been produced by Polymun Scientific in co-operation with the IAM [5,6] since the 1990s. A short biological half-life, the relatively high molecular weight (33 kDa) and the hydrophilic nature of this radical-scavenging enzyme were the limiting factors in tissue protection by systemic administration. In order to enhance the therapeutic effect, we encapsulated rh-Cu/Zn-SOD into liposomes. Liposome enhanced targeting offers the opportunity of increasing the therapeutic index by a prolonged sustained release and offers furthermore an improved delivery through the intact skin [7–9].

The first animal studies with liposomally entrapped rh-Cu/Zn-SOD on burned tissues [10] were performed with liposomes prepared by the conventional ethanol injection technique. Promising results led to preliminary clinical studies applying liposomally entrapped rh-Cu/Zn-SOD onto intact skin for the treatment of Peyronie's disease [11]. At that time, the transition from scientific preparation to industrial-scale production of liposomes had to be made.

To provide acceptance of liposomes as pharmaceuticals, several criteria must be fulfilled. Liposomes must be of defined size and in most cases of unimodal narrow-size distribution. Furthermore, liposomes must have sufficient chemical and physical shelf life. The process used for liposome preparation must be reproducible and process conditions must allow the production of sterile and pyrogen-free liposomes. Furthermore, liposome suspensions have to be produced at reasonable costs.

Therefore, we developed a new technique based on the principles of the ethanol injection technique [12,13], whereby substantial progress was achieved, leading from the conventional batch process to a novel continuous procedure. Herein, the principal item is the crossflow injection module, especially designed for this purpose. This specially conceived unit has the benefit of defined and characterized

* Corresponding author. Polymun Scientific, Immunbiologische Forschung GmbH, Nussdorfer Lände 11, A-1190 Vienna, Austria. Tel.: +43-1-36006-6593; fax: +43-1-3697615.
E-mail address: wagner@edv2.boku.ac.at (A. Wagner).

0939-6411/02/$ - see front matter
PII: S0939-6411(02)00062-0

injection streams and permits liposome manufacture regardless of production scale, as scale is determined only by free disposable vessel volumes.

Previous publications demonstrated that the crossflow injection technique that we have developed meets all of the above-mentioned requirements [14,15]. The present paper describes the entire three-step production process, consisting of encapsulation, separation of non-entrapped protein by continuous crossflow filtration, and retrieval of rh-Cu/Zn-SOD by additional filtration. Results of consecutive lots were compared, based on well-defined quality criteria according to good manufacturing practice (GMP).

2. Materials and methods

2.1. Materials

Dipalmitoyl-phosphatidyl-choline (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 hydration buffer. All these reagents came with analysis certificates.

As the substance to be incorporated, rh-Cu/Zn-SOD from *Escherichia coli* has been used. This therapeutically interesting protein is available at the IAM, produced in clinical-grade quality by the project sponsor 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 crossflow injection technique using the multiple injection mode. The crossflow injection technique is an advancement of the ethanol injection technique [12,13]. The production plant is designed to prepare sterile liposome suspensions without demanding any further end product sterilization. Therefore, all parts of the equipment are qualified for depyrogenization and heat sterilization.

The preparation system consists of the crossflow injection module, vessels for the polar phase (PBS buffer solution and protein solution), an ethanol/lipid solution vessel and a nitrogen pressure device. The crossflow injection module used for liposome manufacture is made of two stainless steel tubes welded together forming a cross. At the connecting point the module has an injection hole (250 μm drill hole) drilled by spark erosion (Heun, Germany).

All reagents, such as the buffer solution, the protein solution and the lipid-ethanol solution, were transferred into the sterilized containment by filtration through 0.22 μm filters (Millipore, Bedford, MA). Nitrogen for the injection process was also filtered through a 0.22 μm filtration unit (Pall, NY). The lipid mixture is dissolved in stirred 95% ethanol at 55 °C. The buffer/protein solution is also tempered at 55 °C. The injection pressure amounted to 2.5 bar.

As shown in Fig. 1, the protein solution was pumped from vessel A to vessel B passing the crossflow injection module, where the ethanol/lipid solution is injected into the protein solution, which is immediately diluted in the stirred buffer solution in vessel B. The final ethanol concentration in the resulting liposome suspension amounted to 7.5%.

2.3. Filtration

Unentrapped protein was separated by crossflow ultradiafiltration equipment (Sartocon Slice cassettes, Polysulfone, cut off: 100 kDa; Sartorius, Germany). This module is distinguished by an excellent chemical and thermal stability, thus sterile filtration conditions are guaranteed. As shown in Fig. 2, the system is designed for continuous diafiltration. All materials were depyrogenized followed by heat sterilization. The PBS buffer solution was filtered through a 0.22 μm filtration device. Parts of the filtration system, which were not autoclavable, like the liposome suspension vessel (vessel 2) and sterile bags, were implemented as sterile equipment, connected in a biological safety cabinet (class II). Protein removal was performed as follows: the liposome suspension was transferred to the main filtration vessel 1, wherefrom it was pumped to the filtration membrane and back to the main vessel. Separated protein solution was transferred to filtrate bag 5 and the removed filtrate volume was automatically replaced by buffer solution from buffer bag 3. Filtration was carried out until unentrapped protein reached less than 100 μg/ml filtrate solution.

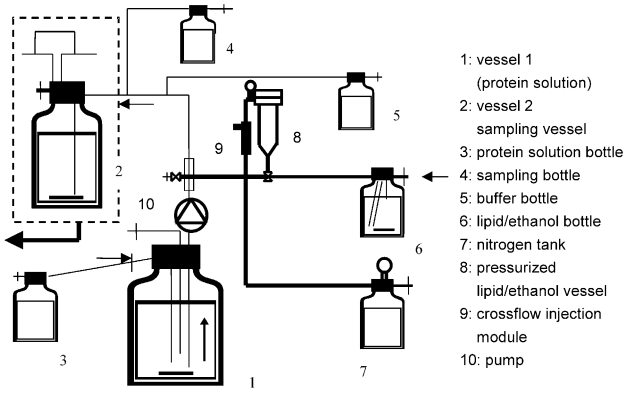

Fig. 1. Schematic sketch of the pilot plant for the production of investigational medicinal liposome batches. The protein solution is pumped from vessel 1 through the injection module, where liposomes are formed. The lipid/ethanol solution is injected with nitrogen pressure (vessel 7 nitrogen tank). Immediately after the injection procedure in the injection module the liposome suspension is diluted with buffer solution in vessel 2.

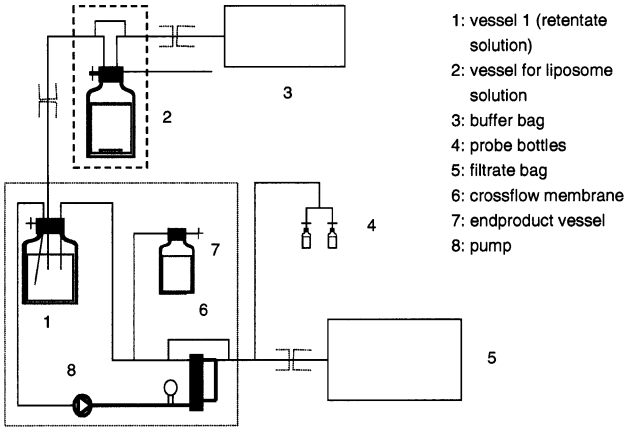

Fig. 2. Schematic sketch of the continuous crossflow filtration plant for the separation of unentrapped protein from the liposome suspension. The liposome suspension in vessel 2 is undocked from the liposome preparation system and implemented into the filtration system. The liposome suspension is transferred to the main filtration vessel 1, wherefrom it is pumped to the filtration membrane 6 and back to the main vessel. Separated protein solution is transferred to filtrate bag 5 and the removed filtrate volume is automatically replaced by buffer solution from buffer bag 3.

2.4. Protein retrieval

The preparations for filtration were made according to the protein removal process. The protein solution was transferred from the protein solution bags to the sampling bottle 5 and filtered with a depth filter (0.8–0.2 μm) to remove lipid residues (Fig. 3). This step was suitable to avoid clogging of the filtration membrane. This was continued by a transfer to vessel 1 and ultrafiltration starts. Removed filtrate volume was replaced by protein solution from vessel 5. After protein concentration the ultrafiltration was followed by a diafiltration step to remove the ethanol residues.

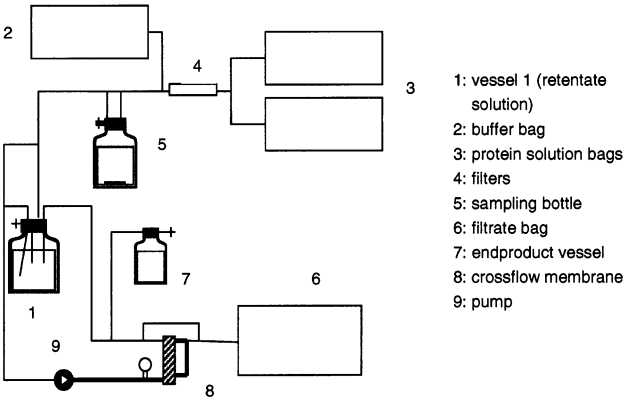

Fig. 3. Schematic sketch of the filtration plant for protein retrieval. The protein solution is transferred from the protein solution bags to the sampling bottle 5 and filtered with a depth filter (0.8–0.2 μm) to remove lipid residues. This is continued by a transfer to vessel 1 and ultrafiltration starts. Removed filtrate volume is replaced by protein solution from vessel 5. After protein concentration the ultrafiltration is followed by a diafiltration step to remove the ethanol residues.

2.5. Analytical methods

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

2.5.1. Liposome size distribution

2.5.1.1. Flow cytometry measurement technique (FCM-technique)

Liposome size and size distribution were measured by a novel technique using flow cytometry [16]. The size determination was performed using a FACS Vantage (Becton Dickinson, San Jose, CA), equipped with a 5 W 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 (700 V, 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.

2.5.1.2. Photon correlation spectroscopy (PCS)

Measurements for the determination of the average particle mean were performed on a Zetasizer 3000 HS (Malvern, Southborough, MA, GB) equipped with a 5 mW helium/neon laser. Experiments were carried out at room temperature with a detection angle of 90° for 200 s each. The calculations of data were performed with the contin mode, where the distribution is adapted to a Gaussian normal distribution and with the exponential sampling method, separating the particle distribution into 24 equidistant classes. The results are given in average mean diameters with an additional polydispersity index, which gives further information about the inhomogeneity due to liposome preparation.

2.5.2. Quantification of entrapped and non-entrapped drug

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

To determine the internal protein content, the liposome sample is separated from unentrapped protein by filtration. In the next step, the sample is diluted with detergent and incubated at 37 °C for at least 120 min to disintegrate the lipid bilayers. This process leads to micelle formation of the membrane compounds, and the encapsulated protein is released [17]. The non-encapsulated 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-step ELISA, in which the immobilized capture antibody is different from the alkaline phosphatase conjugated labelling antibody. After staining, the plates are measured at 405 nm using the reference wavelength of 690 nm. The quantity of

Table 1
Protein encapsulation rates of consecutive lots[a]

	Batch 1	Batch 2	Batch 3	Batch 4
Rh-SOD start concentration (mg)	18000	18000	17600	26400
Entrapped rh-SOD (mg)	4620	4734	4540	7128
% of entrapped rh-SOD	25.6	26.3	25.8	27

[a] Rh-Cu/Zn-SOD amount of liposome production (triple injection) determined by an enzyme-linked immunosorbent assay (ELISA).

incorporated protein can be calculated from the difference of the entire rh-Cu/Zn-SOD content minus the external content.

2.5.3. Protein activity assay

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

2.5.4. Microbiological testing

Microbiological testing for the absence of colony forming units was performed according to European Pharmacopoeia Guidelines 1997, Supplement 2000 (chapters 2.6.12 and 2.6.13).

3. Results

3.1. Liposome production

The liposome suspensions were produced by the cross-flow triple injection procedure, as described in Section 2 and shown in Fig. 1. Through all production batches 25–27% of the present protein was entrapped into liposomes. As shown in Table 1 and Fig. 4a–d, entrapment values were reproducible in all production batches.

Size and size distribution of all liposome batches were determined by the flow cytometry technique established in-house. The production batches, compared in Fig. 4a–d, show the reproducibility of the liposome preparation procedure. The vesicle mean diameter ranged between 270 and 290 nm.

Comparative measurements were performed by PCS and as demonstrated in Fig. 5a,b, both measurement techniques yield similar results. Liposomes determined by PCS resulted in a mean diameter of 281.6 nm having a polydispersity index of 0.25. The same vesicles determined by FCM had a mean diameter of 285 nm (see also Fig. 4a–d).

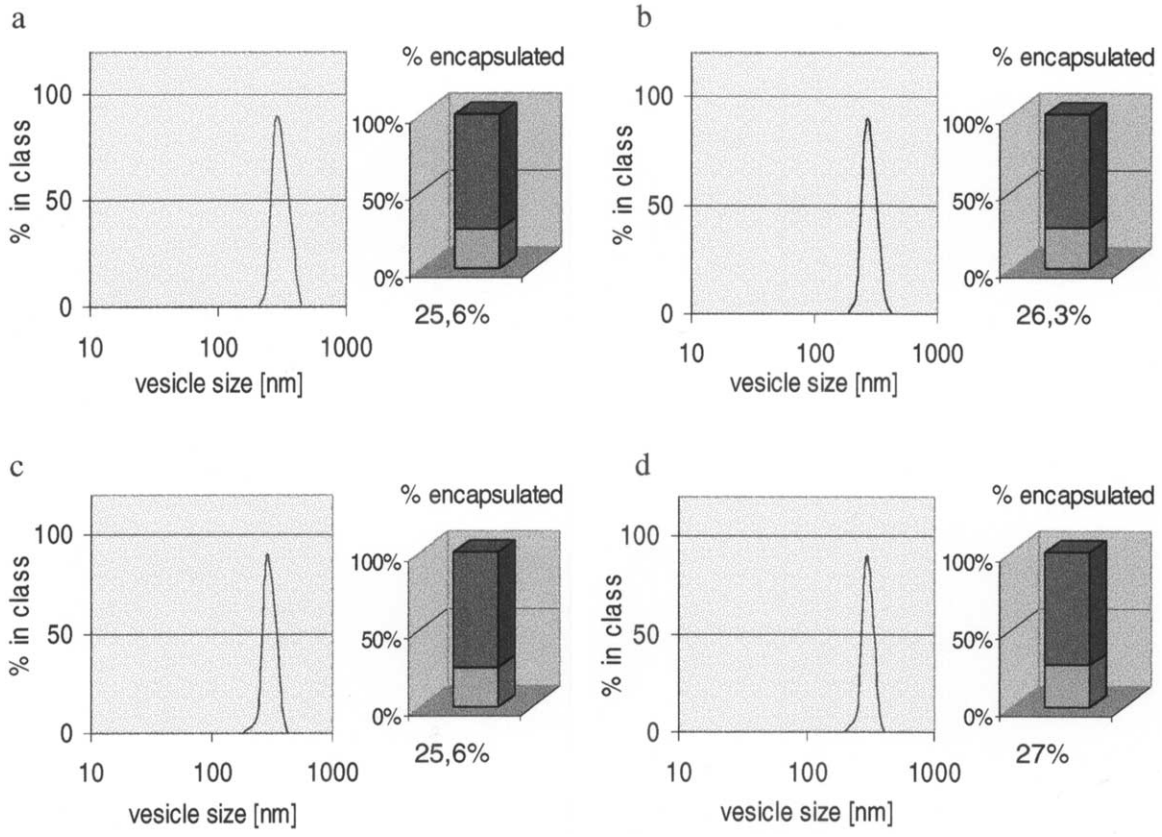

Fig. 4. (a–d) Liposome size and size distribution and encapsulation rates of the consecutive production lots. Vesicle size is determined by the flow cytometry technique and encapsulation rates are measured by ELISA after filtration.

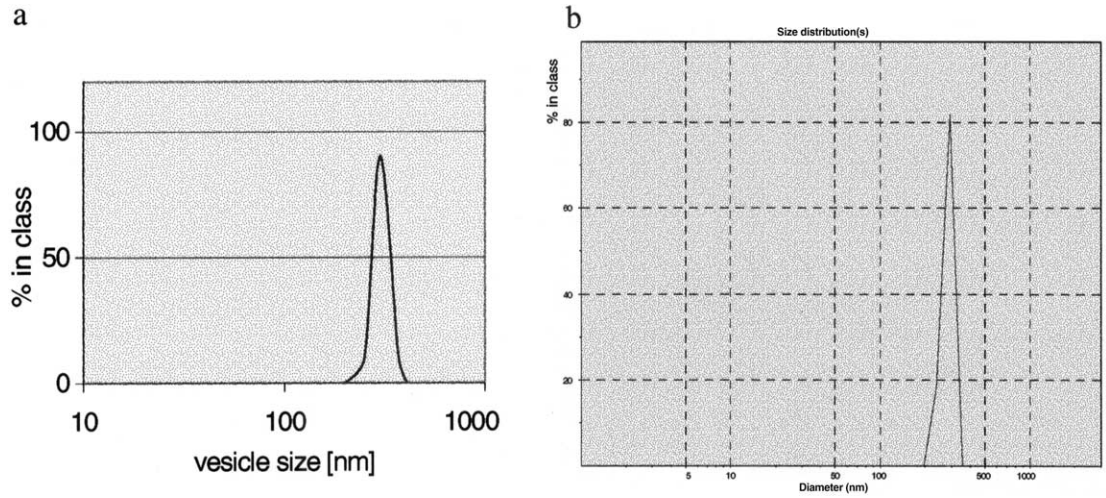

Fig. 5. (a,b) Comparative size measurement of one preparation batch determined by flow the cytometry technique (a) and photon correlation spectroscopy (b).

3.2. Removal of non-entrapped protein

Non-encapsulated rh-Cu/Zn-SOD was removed by continuous crossflow filtration. The filtration plant is described in Fig. 2. The retained liposome suspension (retentate) was concentrated to 3.6 mg liposomally entrapped protein/ml and a final volume of 1250 ml. For the determination of the bilayer stability during filtration, size and size distribution were measured. As demonstrated in Fig. 6, the liposomal structures just slightly changed during the filtration procedure. The vesicle mean diameter increased from 285 to 290 nm.

3.3. Protein retrieval

The filtrates of liposome suspensions were separated to retrieve the protein solution for use in further production cycles. Therefore, a second ultra/diafiltration was performed using a 10 kDa crossflow filtration membrane. The filtration equipment is shown in Fig. 3.

After filtration the enzymatic activity remained stable and about 50–55% of the initial protein content could be retained for further liposome manufacture. Testing for colony forming units showed no microbial contamination.

4. Discussion

Late in the short history of liposomes, in the mid 1980s, the first large scale productions were performed [18–22]. The reason for the relatively slow development of successful large scale production is related to the time-consuming technological problems and quality control issues that had to be resolved, including: (1) availability of high-quality lipid raw materials; (2) validated quality control assays; (3) reproducible processes for large scale production; (4) meth-

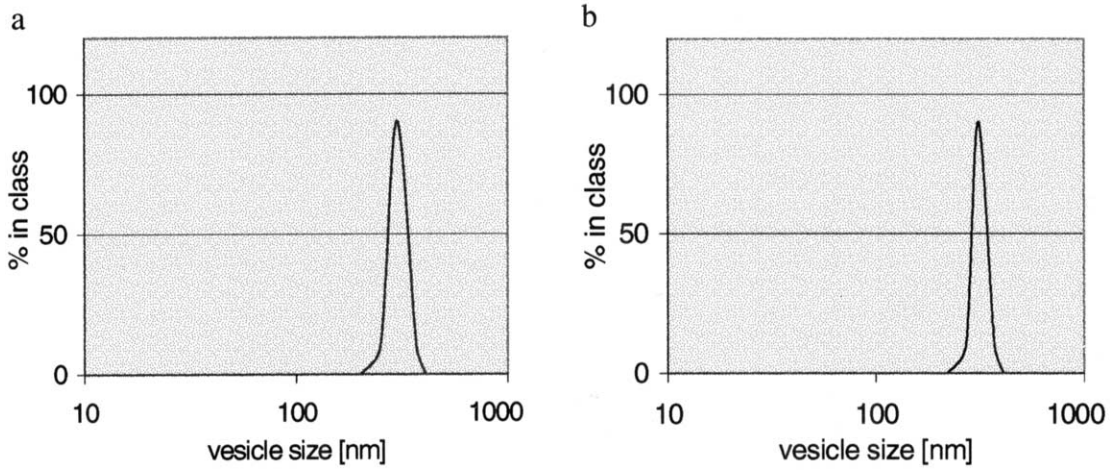

Fig. 6. Liposome size and size distribution of one production batch determined by a flow cytometry technique. The lipid vesicles are compared before (a) and after (b) the filtration procedure.

ods for obtaining sterile liposomal products; and (5) long term chemical and physical stability.

We initiated the development of a drug formulation containing liposomally encapsulated rh-Cu/Zn-SOD, driven by the conviction that liposomally entrapped SOD might have beneficial clinical properties in the treatment of inflammatory diseases [23,24]. For this reason, we focused on the establishment of a manufacturing technique with the potential for economical scale-up.

The goal of our studies was highly reproducible technology for incorporation of pharmaceutically interesting substances into liposomes. Continuous aseptic one-step operation permits the production of stable and sterile liposomes with a defined size distribution. In developing this new technology (unit operation), we achieved substantial progress, leading from the conventional batch process to a novel continuous procedure. This specially conceived unit has the benefit of defined and characterized injection streams and permits liposome manufacture regardless of scale, which is limited only by available vessel volumes. As liposome preparation takes place outside the production vessels, namely in the injection module, this operation ensures scale-up to any desired production size [14].

The production plant is designed to meet several requirements, such as simplicity, robustness and easy handling of sterilization procedures. Furthermore, the ethanol injection technique itself is distinguished by mild preparation conditions and the avoidance of hazardous solvents and forces, which may disrupt lipids as well as entrapped substances. Therefore, we focused on the ethanol injection technique, first described by Batzri and Korn in 1973 [12].

For the passive entrapment of a large and hydrophilic protein into low concentrated liposome suspensions, we designed a sophisticated strategy, which is feasible for our previously established production procedure. In particular for the large scale production of liposomes containing expensive biotechnologically derived substances, a compromise between the therapeutic benefit (entrapment capacity) and economic considerations (entrapment efficiency) has to be taken. The development of the multiple injection technique and increased encapsulation efficiency of water soluble substances have made it possible to produce even large and hydrophilic molecules such as SOD up to 50% of the initial concentration, both in laboratory scale and industrial scale [15]. Hydrophobic drugs can be entrapped as well, resulting in higher encapsulation efficiency.

Subsequently, the potential of this technique has been demonstrated by the production of several consecutive lots. For characterization, size and size distribution, protein encapsulation efficiency, biological activity of the protein, membrane stability and microbial contamination were determined.

As shown in Fig. 4 all production lots show the same size and size distribution. Similar data could be observed in scale-up studies [14]. These data demonstrate impressively the reproducibility and robustness of the established production technique. Consequently, the same amount of admitted protein was entrapped in all four consecutive production batches. Furthermore, as demonstrated in Table 2, the rh-Cu/Zn-SOD, the active therapeutic compound, did not lose any enzymatic activity during the whole production procedure. This is due to the mild and harmless preparation conditions.

The liposomes remained stable during the successive filtration procedure for protein removal. Only minimal alterations with respect to vesicle size and size distribution could be observed. As shown in Fig. 6, size and narrow size distribution remained almost constant. In addition, no loss of protein activity could be detected due to the mild and optimized filtration conditions.

Microbial testing for the absence of colony forming units was performed according to the European Pharmacopoeia guidelines. During the whole liposome manufacture no microbial contamination could be observed.

Table 2
Protein amount and activity during the liposome production process[a]

Process step	Protein concentration (mg)	Enzymatic activity (mg)
Vesicle preparation start concentration	26400	26319
Filtration I: protein removal		
Entrapped protein	7128	6914
Free protein	19000	19228
Filtration II: protein retrieval	14500	14495
Protein losses (transfer, filtration, etc.)	4772	–

[a] Amount and enzymatic activity of rh-Cu/Zn-SOD during all process steps determined by an enzyme-linked immunosorbent assay (ELISA) and an enzymatic activity assay of one preparation batch (except the protein loss, which is calculated from the start amount minus the several process steps).

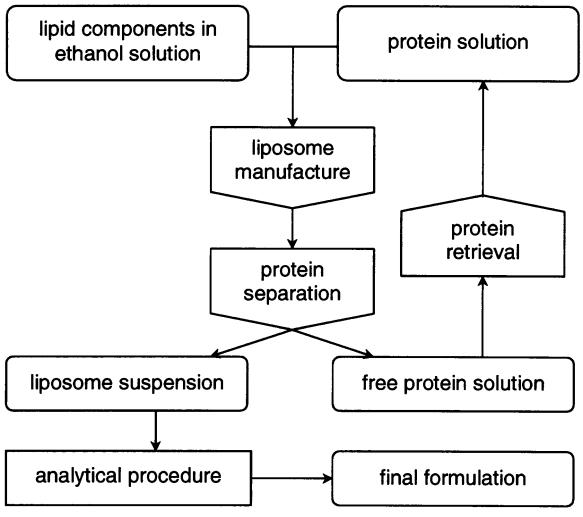

Fig. 7. Flow-chart of the liposome production system for therapeutically applied liposomal rh-Cu/Zn-SOD.

For economic consideration, the retrieval of active rh-Cu/Zn-SOD is an additional advantage of this procedure. Protein solutions, which are suitable for further liposome encapsulation, have to be sterile and free of pyrogens. Furthermore, they have to be free of ethanol and lipid residues and are tested for enzymatic activity. If all these requirements are met, the protein solution is used for further liposome manufacture. The flow-chart in Fig. 7 demonstrates the efficiency of this closed-loop technology.

In conclusion, liposome manufacture with the crossflow technique is suitable for the production of clinically relevant material. The main advantage of this technique is the feasibility of manufacturing batches of 5–10 ml, which is directly scalable to several litres. This variant avoids cost-intensive scale-up and permits early prognosis about product quality, as liposomes can be screened more quickly and more efficiently. This is of particular importance when it comes to cost-intensive drugs that are to be manufactured by novel biotechnological procedures.

Acknowledgements

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

References

[1] E.T. Dams, W.J. Oyen, O.C. Boerman, G. Storm, P. Laverman, P.J. Kok, W.C. Buijs, H. Bakker, J.W. van der Meer, F.H. Corstens, 99mTc-PEG liposomes for the scintigraphic detection of infection and inflammation: clinical evaluation, J. Nucl. Med. 41 (2000) 622–630.

[2] M. Foldvari, A. Gesztes, M. Mezei, Dermal drug delivery by liposome encapsulation: clinical and electron microscopic studies, J. Microencapsulation 7 (1990) 479–489.

[3] A. Gabizon, R. Isacson, E. Libson, B. Kaufman, B. Uziely, R. Catane, C.G. Ben-Dor, E. Rabello, Y. Cass, T. Peretz, Clinical studies of liposome-encapsulated doxorubicin, Acta Oncol. 33 (1994) 779–786.

[4] P. Laverman, A.H. Brouwers, E.T. Dams, W.J. Oyen, G. Storm, N. van-Rooijen, F.H. Corstens, O.C. Boerman, Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose, J. Pharmacol. Exp. Ther. 293 (2000) 996–1001.

[5] K. Bayer, A. Jungbauer, W. Kramer, H. Lettner, W. Schönhofer, M. Skias, F. Steindl, N. Taverner, K. Uhl, Humane rekombinante Superoxiddismutase, Bioengineering 6 (1990) 24–30.

[6] K. Vorauer-Uhl, M. Skias, A. Trkola, W. Schönhofer, F. Unterluggauer, P. Schulz, A. Jungbauer, Flocculation: an alternative process to ion-exchange chromatography: (A scale-up study using recombinant human superoxide dismutase as model protein), Bioseparation 3 (1992) 217–226.

[7] V.B. Patel, A. Misra, Y.S. Marfatia, Topical liposomal gel of tretinoin for the treatment of acne: research and clinical implications, Pharm. Dev. Technol. 5 (2000) 455–464.

[8] M.H. Schmid, H.C. Korting, Liposomes: a drug carrier system for topical treatment in dermatology, Crit. Rev. Ther. Drug Carrier Syst. 11 (1994) 97–118.

[9] M.H. Schmid, H.C. Korting, Therapeutic progress with topical liposome drugs for skin disease, Adv. Drug Deliv. Rev. 18 (1996) 335–342.

[10] K. Vorauer-Uhl, E. Fürnschlief, A. Wagner, B. Ferko, H. Katinger, Topically applied liposome encapsulated superoxide dismutase reduces postburn wound size and edema formation, Eur. J. Pharm. Sci. 14 (2001) 63–67.

[11] R.C. Riedl, E. Plas, K. Vorauer-Uhl, B. Vcelar, A. Wagner, H. Pflüger, A pilot study on liposomal recombinant superoxide dismutase for the treatment of Peyronie's disease, Eur. J. Urol. 40 (2001) 343–349.

[12] S. Batzri, E.D. Korn, Single bilayer liposomes prepared without sonication, Biochim. Biophys. Acta 298 (1973) 1015–1019.

[13] J.M. Kremer, M.W. Esker, C. Pathmamanoharan, P.H. Wiersema, Vesicles of variable diameter prepared by a modified injection method, Biochemistry 16 (1977) 3932–3935.

[14] A. Wagner, K. Vorauer-Uhl, H. Katinger, The crossflow injection technique – an improvement of the ethanol injection method, J. Liposome Res. (2002) in press.

[15] A. Wagner, K. Vorauer-Uhl, G. Kreismayr, H. Katinger, Enhanced protein loading into liposomes by the multiple injection technique, J. Liposome Res. (2002) in press.

[16] K. Vorauer-Uhl, A. Wagner, N. Borth, H. Katinger, Determination of liposome size distribution by flow cytometry, Cytometry 39 (2000) 166–171.

[17] J. Hernández-Borrell, M. Pons, J.C. Juarez, J. Estelrich, The action of Triton X-100 and sodium dodecyl sulphate on lipid layers. Effect on monolayers and liposomes, J. Microencapsulation 7 (1990) 255–259.

[18] T. Schneider, A. Sachse, G. Rößling, M. Brandl, Large-scale production of liposomes of defined size by a new continuous high pressure extrusion device, Drug Dev. Ind. Pharm. 20 (1994) 2787–2807.

[19] R. Naeff, Feasibility of topical liposome drugs produced on an industrial scale, Adv. Drug Deliv. Rev. 18 (1996) 343–347.

[20] S. Amselem, A. Gabizon, Y. Barenholz, A large-scale method for the preparation of sterile and nonpyrogenic liposomal formulations of defined size distributions for clinical use, in: G. Gregoriadis (Ed.), 2nd Edition, Liposome Technology, 1, CRC Press, Boca Raton, FL, 1993, pp. 501–525.

[21] H.G. Weder, Liposome production: the sizing up technology starting from mixed micelles and the scaling up procedure for the glucocorticoid bethamethson diproprionate and bethamethasone, in: O. Braun-Falco, H.C. Korting, H.I. Maibach (Eds.), Liposome Dermatics, Springer-Verlag, Berlin, 1992, pp. 101–109.

[22] R.W. Kriftner, Liposome production. The ethanol injection technique and the development of the first approved liposome dermatic, in: O. Braun-Falco, H.C. Korting, H.I. Maibach (Eds.), Liposome Dermatics, Springer-Verlag, Berlin, 1992, pp. 91–100.

[23] C.M. Regnault, M. Roch-Arveiller, M. Tissot, Effect of encapsulation on the anti-inflammatory properties of superoxide dismutase after oral administration, Clin. Chim. Acta 240 (1995) 117–127.

[24] A. Garcia-Gonzalez, J.L. Ochoa, Anti-inflammatory activity of Debaryomyces hansenii Cu,Zn-SOD, Arch. Med. Res. 30 (1999) 69–73.