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Research paper

Non-phospholipid vesicles for pulmonary glucocorticoid delivery

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Abstract

In the formulation of inhaled drugs for the treatment of asthma and chronic obstructive pulmonary disease (COPD), considerable attention has been devoted to new aerosol morphologies which can either enhance the local effect and/or increase the penetration through the mucus, secreted in bronchial inflammatory diseases. In diseases characterized by bronchial hypersecretion, lipophilic substances, such as corticosteroids, can be remarkably impeded in reaching their receptors, which are localized within the cytoplasm of bronchial epithelial cells. Vesicles consisting of one or more surfactant bilayers enclosing aqueous spaces, are of particular interest because they offer several advantages with regard to chemical stability, lower cost and availability of materials compared to conventional liposomes. With the purpose of carrying out research leading to an innovative formulation for lung delivery capable of permeating the mucous layer, beclomethasone dipropionate, clinically used for the treatment of asthma and COPD, was entrapped in non-phospholipid vesicles. The composition providing the highest entrapment efficiency was chosen. The vesicles obtained after jet nebulization were characterized by means of freeze-fracture microscopy and dynamic light scattering. The efficiency of this new drug delivery system was evaluated in vitro with simulated mucus by means of diffusion experiments (three compartment cell apparatus), using 0.1% mucin gel-like dispersion as a barrier to drug permeation.

Keywords: Non-phospholipid vesicles; Lung delivery; Beclomethasone dipropionate; Asthma; Chronic obstructive pulmonary disease; Mucus permeation

1. Introduction

The basis for the success of pharmacotherapy is the design of an appropriate delivery system, that targets the drug to its site of action, at an optimum concentration and for a determined time interval.

Inhaled glucocorticosteroids (ICS) are the most effective therapy available for patients with asthma. Although, the beneficial effects of ICS on patients with asthma have been conclusively demonstrated, the role of these drugs in the management of chronic obstructive pulmonary disease (COPD) is less certain [1–5]. This probably relates to a number of factors including mucosal hypersecretion that is perhaps more marked in COPD patients, in addition to bronchospasm and mucosal edema that is present in both conditions.

Glucocorticoids are lipophilic substances and do not easily permeate through the hydrophilic mucus in order to reach their site of action, the glucocorticoid receptor, located in the cytoplasm of bronchial epithelial cells [6].

Aerosolized liposomes offer the additional advantages of targeted drug delivery and amplified therapeutic effect [7–9]. Among different aereosol delivery technologies, nebulizers represent a simple device because vesicular structures may be delivered without further processing as for metered doser inhalers or dry powder inhalers [10–12]. On the other hand, the nebulization of liposomes exhibits some difficulties, including physical and chemical instability, before and after nebulization, a relatively low drug

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load-carrying capacity and high costs of production. An alternative approach, that overcomes several of these problems, involves the use of non-ionic surfactants for the formation of bilayer vesicles [13,14].

Beclomethasone dipropionate (BDP), a water insoluble glucocorticoid, is often delivered to the respiratory tract as an aerosol. In this case, the vesicular structures may represent an appropriate system for the solubilization of the relatively insoluble drug, for a sustained pulmonary delivery and, at the same time, because of the presence of a surfactant, for a more efficient permeation through the mucous barrier in COPD.

In order to evaluate the possible advantages of a recently proposed new type of non-phospholipid vesicle system for pulmonary drug delivery that can lead to an improved mucus permeation, we encapsulated BDP, as a reference-model drug, in vesicular structures obtained with polysorbate 20. The aim of this study was to evaluate 'in vitro' the effectiveness of such delivery system that should enhance permeation through mucosal barriers because of the presence of vesicles formed with a remarkably hydrophilic non-ionic surfactant usually considered as unsuitable for the formation of vesicular structures because of its high HLB value(HLB = 16.7) [13].

2. Materials and methods

2.1. Materials

The non-ionic surfactant (polysorbate 20) was a Merck (Germany) product. Cholesterol USP was provided by Carlo Erba (Italy). Mucin and Hepes salt ({*N*-(2-idroxyethyl) piperazine-*N*′-(2-ethanesulfonicacid)}) were Sigma-Aldrich (Italy) products, BDP and the commercial product (Clenil per Aerosol) were the generous gifts of Chiesi Farmaceutici (Italy). Sephadex G 75 was purchased from Pharmacia (Italy). All other products and reagents were of analytical grade. Distilled water was always used. Viscosity of analyzed samples was measured with a rheometer (Rheo-Tec, Radeburg, Germany). Nebulization was carried out using a TurboBoy (Pari, USA) and a Clenny (Chiesi, Italy) apparatus.

2.2. Preparation of non-ionic surfactant vesicles

Unilamellar vesicles were obtained from a non-ionic surfactant/BDP aqueous dispersion (Hepes pH 7.4) by means of the 'film' method as previously reported [13,15], according to the compositions reported in Table 1. For this purpose polysorbate 20, cholesterol and BDP were dissolved in a $CHCl_3/CH_3OH$ (3:1) mixture in a round-bottomed flask. After evaporation of the solvents, the dried film was hydrated by addition of 5 ml (0.01 M) Hepes pH 7.4 solution. The dispersion was vortexed for about 5 min and then sonicated for 5 min at 60 °C using a tapered microtip operating at 20 kHz at an amplitude of 16% (Vibracell-VCX 400-Sonics, USA). For an appropriate control, vesicles without BDP were also prepared.

Table 1
Sample composition expressed as % w/v

Sample	Polysorbate 20	Cholesterol	BDP
1	1.84	0.58	0.5
2	1.84	0.58	1.0
3	1.84	0.58	3.0
4	1.84	0.58	5.0
5	3.68	1.16	0.5
6	3.68	1.16	1.0
7	3.68	1.16	3.0
8	3.68	1.16	5.0

2.3. Vesicle purification

In order to separate drug-loaded vesicles from untrapped substances, for the evaluation of entrapment efficiency, the vesicle dispersion was purified by gel-filtration on Sephadex G75 (glass column 50 × 1.2 cm), using Hepes buffer as eluent. Twenty millilitre of vesicle dispersion were collected. According to a quantitative evaluation [16] of the polysorbate 20 carried out on a purified preparation, the percentage of surfactant actually structured to form the vesicles was determined to be 41%.

2.4. Sample characterization

2.4.1. Freeze-fracture microscopy technique

For vesicle characterization, before and after nebulization, the samples were examined by means of the freeze-fracture microscopy technique: samples were impregnated in 30% glycerol and then frozen into partially solidified Freon 22, freeze-fractured in a freeze-fracture device (−105 °C, 10^{-6} mmHg) and replicated by evaporation from a platinum/carbon gun. The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a Philips CM 10 transmission electron microscope (Fig. 1A and B).

2.4.2. Size measurements, zeta potential and stability tests

Size measurements, before and after nebulization carried out with the jet nebulizer, and evaluation of vesicle stability were carried out by means of dynamic light scattering. The vesicle dispersions were diluted about 100 times in the same buffer used for their preparation. Dust particles were eliminated by filtration (0.45 μm) from the buffer solution as well as from the vesicle preparation. Vesicle size distribution was measured on a Malvern Nano ZS90 (Malvern, UK) at 25 °C, with a scattering angle of 90.0°. The same apparatus was used for the evaluation of zeta potential using a vesicle preparation appropriately diluted (1:10) in distilled water at 25 °C. The polidispersity index

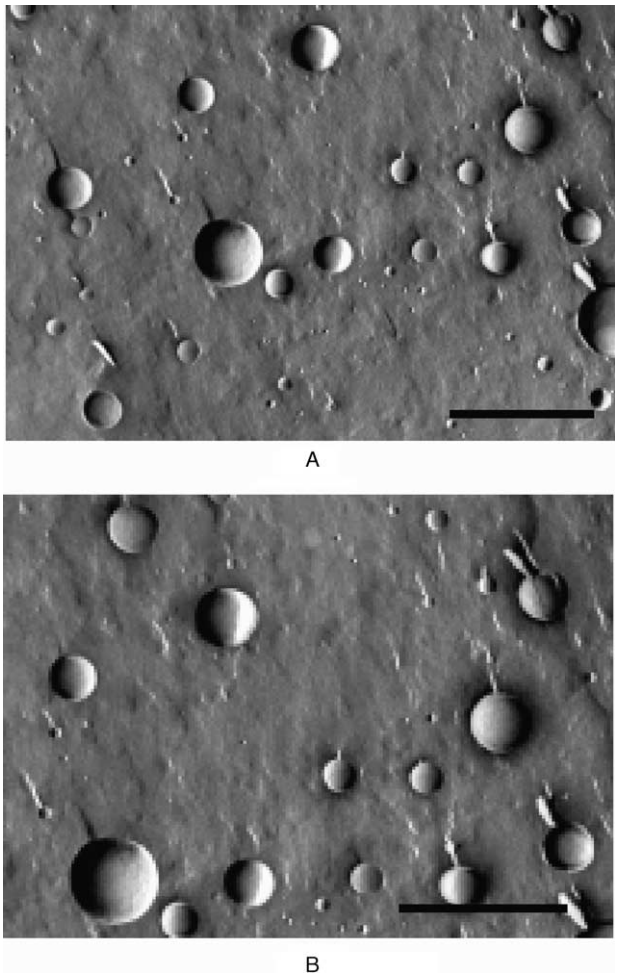

Fig. 1. Trasmission electron micrographs of BDP-loaded TNI vesicles after freeze-fracture, before (A) and after (B) nebulization. The scale bar represents 0.5 μm.

was directly calculated by the software of the apparatus and the values obtained are in agreement with mono disperse vesicular systems. Vesicle stability, in terms of changes in vesicle dimensions after aggregation, was evaluated using the same technique on samples stored, up to 1 month, at 4, 25 and 37 °C. This type of information was also confirmed by means of zeta potential measurements.

2.4.3. Determination of drug entrapment efficiency

BDP entrapment within the vesicles was assessed by HPLC on purified vesicles, after their disruption with isopropanol (vesicle dispersion/isopropanol 1/1). All analyses were carried out on a Perkin–Elmer 250 liquid chromatography apparatus, equipped with a Perkin–Elmer 235 photo-diode array detector, a 20 μl Rheodyne injector and a computer integrating apparatus. The column was a Supelcogel ODP-50 (15 cm×4.00 mm I.D.); the mobile phase was a 80/20 mixture of methanol/water. The flow rate was 1.0 ml/min, and BDP was detected at 255 nm.

Drug encapsulation efficiency (e.e.) was calculated according to Görner et al. [17].

$$\text{e.e.} = 100 \times \frac{\text{mass of incorporated drug}}{\text{mass used for vesicle preparation}} \quad (1)$$

2.5. Nebulization of vesicle dispersions

For the particle size measurement of the aerosolized droplets an API Aerosizer Mach 2 (Amherst, MA, USA) was used. After nebulization, the mass median aerodynamic diameter (MMAD) of aerosol droplets was determined from the cumulative mass distribution in the API Aerosizer, at 18 °C and at 60% of relative humidity. Nebulization of the un-purified vesicle dispersion was done with TurboBoy (Pari) and Clenny (Chiesi) jet nebulizers that show superior performance as assessed in previous studies [18,19]. A volume fill of 2 ml was used for the nebulization. The nebulization efficiency (N.E.) of a vesicular formulation is defined as the total output of drug collected on filters calculated as a percentage of the total amount submitted to nebulization. Thus N.E., according to Desai et al. [20], was determined as

$$\text{N.E.}(\%) = \frac{\text{Aerosolized drug (i.e. collected on the filters)}}{\text{Total drug placed in the nebulizer}} \times 100 \quad (2)$$

For this purpose each preparation was nebulized on a paper filter that was extracted with methanol for quantitative BDP determination by HPLC (Fig. 3), as reported above.

2.6. In vitro permeation experiments

In vitro permeation experiments were performed in a three compartment diffusion cell [21]; the central compartment, separated from the outer ones by cellophane membranes (cut-off: 12,000 Da) and simulating the mucosal barrier to permeation, contained a 0.1% (w/v) mucin solution. This was chosen because glycoproteins are the major components of the protein fraction of mucus. Permeation experiments were carried out for 24 h, at 37 °C, through gel-like mucin solution, under continuous stirring, using un-purified vesicle preparations. The exact half cell volumes (3.2 ml) and surface areas (0.64 cm^2) were taken into account when analyzing permeation data. The receiver content was 1/1 ethanol/pH 7.4 buffer, in order to ensure pseudo-sink conditions. Before each experiment, the system was allowed to equilibrate for 1 h. At fixed time intervals, 50 μl samples were drawn from the acceptor compartment and BDP concentration was determined by HPLC, as described above, and an equivalent amount of

ethanol/buffer solution was added each time to maintain a constant volume in the receiver compartment. Dilution of the receiver medium was taken into account when processing the permeation data. The donor compartment contained the same vesicle/BDP preparation used for nebulization experiments. For an appropriate comparison, a BDP/polysorbate 20 dispersion (i.e. with the non-ionic surfactant in its micellar state) was used, as well as a commercial product (Clenil per Aerosol), at the same drug concentrations. The drug diffusion rate through mucin, using vesicular systems, was compared with the permeation profiles obtained using the other samples, i.e. drug/surfactant dispersions and the commercial BDP product. The cumulative amount of BDP that permeated through the mucin solution after the *n*th sampling (Q_n) was estimated by Eq. (3) [22]

$$Q_n = C_n V + \sum_{i=1}^{n-1} V_s C_i \quad (3)$$

where C_i and C_n are the various measured concentrations from 1 to n, V is the volume of the solution in the acceptor compartment and V_s is the sampling volume.

2.7. Statistical treatment

Each system was tested in two different sets of experiments and, within each set, the experiments were repeated three times. The obtained data were compared using the Mann–Whitney test, the Kruskall Wallis test (non-parametric ANOVA) and the Dunnis multiple comparison test. In all cases, the P values were significant ($P < 0.05$).

3. Results and discussion

Size measurement experiments indicate that BDP-loaded vesicles are slightly larger than empty ones as reported in Table 2 for samples 2 and 8 of Table 1, there is an increase in diameter between 10 and 20%, and this expected effect can be related to drug partition between the bilayer and the aqueous core of the vesicles. Accordingly, the presence of BDP in the formulation may affect zeta potential values; as it is possible to observe from Table 2, the corresponding samples 2 and 8 show a significant decrease in zeta potential that approaches the value obtained with BDP alone. This effect can be related to the chemical steroidic structure of the drug that is somehow similar to that of cholesterol and allowing it to fit well within the vesicular structure. Furthermore, it should be pointed out that electron microscopy carried out on numerous samples (10) indicated that nebulization does not influence drug-loaded vesicle dimensions (Fig. 1A and B).

Analyzed samples showed a good stability in terms of possible changes in vesicle dimensions after aggregation. Size measurement experiments indicated that after 1 month at 25 °C, no appreciable vesicle dimension variations could be detected.

The best entrapment efficiency was obtained for sample 8 and the calculated drug e.e. indicated that only about 20% of the overall amount of BDP is actually enclosed within the vesicles. This result is in agreement with the data reported by previous authors [23,24]. For this reason, for permeation and nebulization experiments, the formulation corresponding to sample 8 of Table 1 was used.

We also evaluated the possibility of using the novel vesicular dispersion in a conventional jet nebulizer widely used in clinical applications. For this purpose, samples were characterized also by means of rheological measurements and the aerodynamic diameter was determined (Table 2) as well as the nebulizer mass output (Fig. 2) after completion of nebulization. Evaluation of MMAD (2.0 ± 0.2 μm) and of geometric standard deviation (GSD)

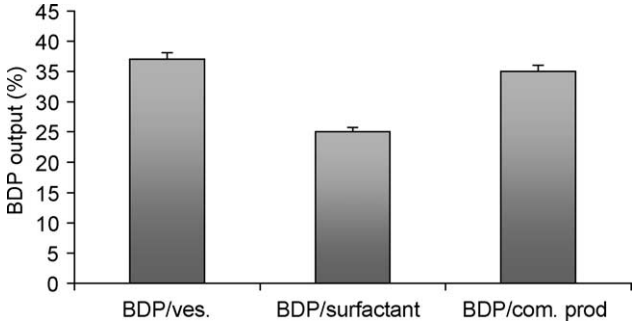

Fig. 2. Deposition of vesicle-encapsulated BDP(BDP/ves) on filters upon nebulization, compared to a BDP/surfactant solution and to a BDP commercial product ($n = 3$, ±SD).

Table 2
Vesicle dimensions (nm) and zeta potential (mV) of analyzed samples ($n = 3$; ±SD)

Sample	Dimensions (nm)	Zeta potential (mV)
2	163 ± 0.03	−32 ± 0.2
8	174 ± 0.02	−34 ± 0.3
Empty vesicles	146 ± 0.05	−40 ± 0.2
BDP solution 0.05% w/v		−30 ± 0.1

Table 3
Percentage of particles with aerodynamic diameter <10, <5, <2 μm, containing non-ionic surfactant vesicles, delivered by a jet nebulizer

Aerodynamic diameter	Percentage
<10	100
<5	99.5
<2	65

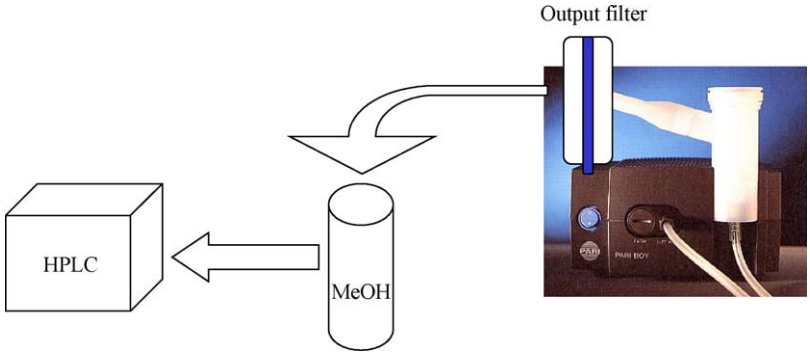

Fig. 3. Device for nebulization efficiency determination: aerosolized particles leaving the nebulizer were captured by a filter; drug amount, extracted by the filters with methanol, was determined by HPLC.

were also carried out; the GSD value (1.5) demonstrates the polydisperse nature of the distribution of the aerosolized droplets that, on the other side, contained a monodisperse vesicular system.

Drug viscosity may affect the aerosol output, i.e. when drug viscosity increases, it causes a decrease in aerosol output. Therefore, it is essential to acquire information about the viscosity of the preparation that must be aerosolized. We found that viscosity of the novel BDP vesicle formulation is comparable to that of commercial products. Clenil® (the BDP formulation by Chiesi Farmaceutici, Parma, Italy) shows a viscosity of 2.5 mPa; the corresponding values for the commercial BDP buffer dispersion and the BDP non-ionic vesicle formulation were, respectively, 2.6 and 2.2 mPa.

The clinical efficacy of therapeutic aerosols depends on the mass delivered to the lungs in suitably small particles capable of reaching the appropriate airway receptors [25]. This fine particle fraction (FPF) consists essentially of particles with an aerodynamic diameter between ∼0.5 and 5 μm. The mass fraction of particles in this range is determined by measuring the aerodynamic size distribution of the aerosol and the drug output from the nebulizer [26] The obtained data (Table 3) show that the percentage of FPF is over 99.5%. This result, together with the drug mass output evaluation (Fig. 2), determined by HPLC from the nebulized fractions (Fig. 3), should ensure a suitable dose for therapeutic efficacy. Results reported in Fig. 2 refer to the experiments performed with the TurboBoy nebulizer, one of the most efficient jet nebulizers. Experiments with a Clenny nebulizer were also carried out; the results observed with this second device were always similar to those obtained with TurboBoy. Actually, in the case of Clenny a slightly greater amount of drug (about 5%) on filters was detected, for all tested samples. In all conditions of nebulization, the dispersion BDP/vesicles seems to release a greater amount of drug on filters with respect to the other formulations.

In Fig. 4, the permeation rate of BDP from the vesicular dispersion is reported and compared with that obtained using a BDP/polysorbate 20 (at the same concentration used for vesicle preparation) suspension as well as with that of the commercial preparation (Clenil®). The vesicular formulation (8 of Table 1) was used in its un-purified form, i.e. not passed through Sephadex G75, thus with the drug partitioned inside and outside the vesicular structure. This situation allows an appropriate comparison with the other preparations used in permeation experiments: since both micellar surfactant solutions and the commercial Clenil® contained free BDP and BDP included within aggregated structures (micelles).

As it can be observed, the presence of non-ionic surfactant vesicles in the formulation remarkably increases the permeation rate through the model mucosal barrier with respect to the other tested preparation thus indicating that the novel BDP formulation can be proposed for a better targeting of corticosteroids in the treatment of COPD.

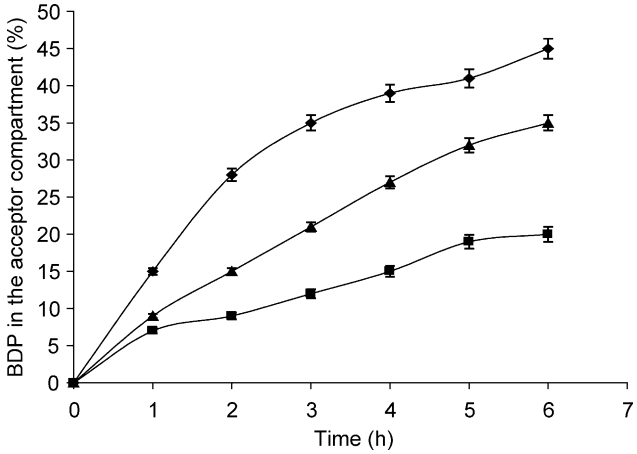

Fig. 4. Comparison of the permeation patterns through a gel-like mucin solution (0.1%w/v), expressed as percentage of permeated drug as a function of time ($n=3$, ±SD) (■ = BDP/surfactant, ▲ = BDP/commercial product, ♦ = BDP/vesicles).

4. Conclusion

From these results, it can be concluded that the vesicular dispersions show rheological characteristics and N.E. (i.e. aerodynamic diameter and drug mass output) comparable with the commercial product.

Furthermore, the novel preparation, according to the aim of this research, ensuring a better penetration through the mucus layer, can provide an efficient system for the treatment of obstructive pulmonary diseases.

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