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Non-phospholipid vesicles as carriers for peptides and proteins: Production, characterization and stability studies

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Abstract

In the present study, the preparation, characterization and activity of non-phospholipid vesicles (NPV) containing three aminoacid-based molecules were described. As model compounds trypsin, bovine basic pancreatic inhibitor and polylysine rich peptides derived from the herpes simplex virus type 1 (HSV-1) glycoprotein B were employed. NPV were chosen as alternative to liposomes for the possible administration of aminoacid based molecules via mucous membrane (nasal or vaginal) routes. NPV containing the indicated model drugs have shown to be more stable in term of size with respect to liposomes encapsulating the same model drugs previously produced by our group [Cortesi, R. Argnani, R., Esposito, E., Dalpiaz, A. Scatturin, A., Bortolotti, F., Lufino, M., Guerrini, R., Incorvaia, C., Menegatti, E., Manservigi, R., 2006. Cationic liposomes as potential carriers for ocular administration of peptides with antiherpetic activity. Int. J. Pharm. 317, 90–100]. In addition our study indicates that the produced NPV (i) are able to encapsulate the model drugs over 49%, (ii) are characterized by dimensions compatible with applications on the mucous membrane, (iii) remain stable in size for at least 3 months and (iv) can release the model drug (after a slight lag time) in a controlled fashion as compared to that of the corresponding free solution.

Keywords: Vesicles; Peptide; Protein; Delivery; Niosomes

1. Introduction

Non-phospholipid vesicles (NPV) have been largely proposed as drug delivery systems (Uchegbu and Vyas, 1998). This class of vesicles appear to be similar in terms of their physical properties to liposomes, being prepared in the same way and under a variety of conditions, forming unilamellar or multilamellar structures (Uchegbu and Florence, 1995). They may be regarded either as inexpensive alternatives, of non-biological origin, to liposomes, or perhaps in vivo as a carrier system physically and structurally very similar to liposomes. Particularly, these vesicles were introduced in reason of the higher chemical stability of the surfactants compared to that of phospholipids. Phospholipids are in fact subjected to stability problems due to the easy hydrolysis of esters bonds or to the possible peroxidation of unsaturated bonds. Moreover, another disadvantage related to the nature of liposomes is referred to the unreliable reproducibility of the lecithin batches. This series of problems in the use of liposomes as drug carriers suggested to the researchers the utilization of alternative sources to make vesicles and among them the use of non-ionic surfactants.

An increasing number of non-ionic surfactants have been found to form vesicles able to entrap hydrophobic and hydrophilic solutes (Uchegbu and Vyas, 1998). Particularly, NPV have been prepared from polyglycerol alkyl ethers (Baillie

Abbreviations: NPV, non-phospholipid vesicles; TRYP, trypsin; BPTI, bovine basic pancreatic trypsin inhibitor; POT, polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate non-phospholipid vesicles; POTC, polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate/cholesterol non-phospholipid vesicles; POS, polyglycero-6-oleate/sorbitan monolaurate non-phospholipid vesicles

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et al., 1985), glucosyl dialkyl ethers (van Hal et al., 1992), crown ethers (Echoyen et al., 1988) and polyoxyethylene alkyl ethers (Hofland et al., 1991).

In the present paper, we investigated the preparation and characterization of NPV based on polyglycerol alkyl ethers and on sorbitan monoalkyl ethers for the transport of aminoacid based molecules, such as trypsin, inhibitor of Kunitz and DTK.

Trypsin (TRYP), is a digestive proteinase, produced in an inactive form by the pancreas, able to break down dietary protein molecules to peptides and amino acids (Schwert and Takenaka, 1955; Pavlisko et al., 1997). The peak of enzymatic activity of TRYP is attained in the small intestine where a slightly alkaline environment (about pH 8) allows pursuit of the digestion process started in the stomach. Among the proteolytic enzymes, TRYP is the most discriminating, since it can attack only a restricted number of chemical bonds. In fact, the presence of residues of hystidine and serine in the catalytic site of TRYP, gives this enzyme the specificity to cleave peptide bonds in protein molecules to which carboxyl groups are donated by arginine and lysine (Pavlisko et al., 1997).

The pancreatic Kunitz inhibitor, also known as bovine basic pancreatic trypsin inhibitor (BPTI), aprotinin and trypsin-kallikrein inhibitor, is one of the most extensively studied globular proteins. It has proved to be a particularly attractive and powerful tool for studying protein conformation as well as molecular bases of protein/protein interaction(s) and (macro) molecular recognition. Moreover, BPTI inhibits the nitric oxide synthase type-I and -II action and impairs K + transport by Ca^{2+}-activated K^+ channels (Ascenzi et al., 2003). The use of BPTI in cardiopulmonary surgery and orthotoptic liver transplantation can significantly reduce hemorrhagic complications and thus blood-transfusion requirements.

DTK is a polylysine rich peptide derived from the herpes simplex virus type 1 (HSV-1). Particularly, this 15 aminoacids residues peptidic sequence (D-T-K-P-K-K-N-K-K-P-K-N-P-P-P) belongs to the extracytoplasmatic region of glycoprotein gB involved in the recognition of a cellular receptor, namely heparan sulfate (HS) proteoglycans, and have been shown to mediate the first binding of the virion to the cell (Shieh et al., 1992; Laquerre et al., 1998). Mutants deleted of this sequence are impared in binding and penetration of the virus into the cell (Laquerre et al., 1998). This polylysine rich sequence, synthesized by our group (Cortesi et al., 2006), has demonstrated to possibly induce neutralizing antibody when injected into rabbits and therefore can be considered a promising immunogen for the development of a new formulated vaccine against HSV infection (Cortesi et al., 2006).

2. Materials and methods

2.1. Chemicals

Polyglycero-6-oleate (Plurol oleique, PO) was from Gattefossè, (Milano, Italy). TRYP, BPTI, cholesterol (C), polyoxyethylen sorbitan monolaurate (T) and sorbitan monolaurate (S) were obtained from Fluka (Buchs, Switzerland). DTK peptide was synthesised by Dr. Remo Guerrini at the department of Pharmaceutical Sciences of Ferrara University.

2.2. NPV preparation

Empty and aminoacid-based molecules containing NPV were prepared as follows: 10 mg of polyglycero-6-oleate/sorbitan monolaurate (POS) (1:1, w/w), polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate (POT) (1:1, w/w), polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate/cholesterol (POTC) mixture (10:10:1, w/w/w), dissolved in 2.0 ml of a methylene chloride/methanol mixture (1:1, v/v) were placed in a 100 ml round bottom flask. After solvent evaporation by vacuum-dried under nitrogen using a rotary evaporator, the resulting dried lipid-film was re-hydrated with 2.0 ml of warm (60 °C) 0.1 M phosphate buffer pH 7.4 with or without peptide-based compound (1 mg/ml) then the mixture was vortexed and sonicated at 60 °C for 10 min in a bath sonicator (Branson 2200, Branson Europe, The Netherlands) to obtain a more homogeneous sized vesicles population (Perkins et al., 1988; Cortesi et al., 1998). Afterwards, the obtained NPV preparation was left at room temperature under continuous stirring (180 mot/min) for 16–18 h in order to give a higher encapsulation efficiency (Leong et al., 2002).

TRYP- and BPTI-containing NPV were then injected into a device (Lipex Biomembranes Inc., Vancouver, Canada), which allowed the extrusion of the vesicles through standard 25 mm diameter polycarbonate filters with calibrated pore size (Nucleopore Corp., Pleasanton, CA). The vesicles were extruded through two stacked filters employing nitrogen pressure of 10–20 bars, collected and re-injected five times. In order to separate the possibly present free model drug from NPV, the turbid suspension was subjected to ultrafiltration. Particularly, 300 μl of each NPV preparation were filtered through 10,000 NMWL or 3000 NMWL pore filters (Micron YM-10, Millipore Corporation, Bedford, USA) by centrifuging at 10,000 rpm for 20 min in a Beckman Microfuge 18 centrifuge. The drug concentration in the preparation (P_T) and in the ultrafiltrate (P_U) was assayed by HPLC as described below. Thus, the equation for the encapsulation efficiency in the liposomes was as follows:

$$\text{encapsulation efficiency}(\%) = \left[\frac{(P_T - P_U)}{P_T}\right] \times 100.$$

Each model drug concentration was determined as reported below.

2.3. Trypsin determination

The structural integrity of TRYP released from NPV was characterized by HPLC using a size-exclusion chromatography (SEC) column. More specifically, 30 μl of the TRYP solution was injected on a TSK-Gel G3000SWXL 7.8 mm × 300 mm column (TosoHaas, United States) and eluted isocratically with 10 mM phosphate monohydrate buffer at pH 4.3. The enzymatic activity of free and NPV-TRYP was determined by spectrophotometric analysis, according to a modified version of the method described by Huber and Bode (1978). Fifty microlitres of non-

phospholipid vesicles containing TRYP sample were added to 1 mg/ml *N*-αbenzoyl arginine (BAPNA) in 200 mM triethanolamine hydrochloride pH 7.8 (1.95 ml) at 25 °C, and progress of the reaction was followed spectrophotometrically at 405 nm, using a Lamba19 Perkin-Elmer UV–vis spectrometer (UK). The specific activity was calculated in BAPNA-units/mg trypsin, 1 BAPNA-unit corresponding to 0.001 DA:min. The activity of untreated trypsin was measured using the same procedure. The values reported are the averages of at least two measurements, the standard deviation being two percentage units. As standard solution (reference) 50 μl of crystallized bovine trypsin (0.2 mg/ml) in HCl 0.001 N were used.

2.4. BPTI determination

The determination of BPTI was obtained as follows. Fifty microlitres of a trypsin standard solution (0.2 mg/ml) were added to 15 μl of NPV containing BPTI and 1935 ml of triethanolamine hydrochloride 0.2 M pH 7.8. After 3 min (time needed to obtain the complete inhibition of trypsin activity by BPTI) 1 ml of BAPNA was added and the analysis was performed as previously described for TRYP determination.

From the comparison with a standard curve obtained by using a concentration of 10 μg/ml of BPTI as a reference, it was possible to obtain the percentage of trypsin inhibition.

2.5. DTK determination

The HPLC determination of DTK was performed using a Jasco (Japan) gradient chromatographic pump, a Rheodyne 7125 sample injection valve (equipped with a 50-μl loop) and a Jasco UV detector. Samples were eluted on a 150 mm × 4.6 mm reverse-phase stainless steel column packed with 5 μm particles (Model BDS C18 Hypersil, Hewlett Packard, USA). The elution was isocratically performed at room temperature with a mobile phase constituted for 86% of water, 14% acetonitrile and 0.15% TFA at a flow rate of 0.8 ml/min. DTK was monitored at 225 nm, its characteristic λ_{max}.

2.6. NPV morphology

NPV shape and surface characteristics were studied by atomic force microscopy (AFM) and cryo transmission electron microscopy (Cryo-TEM).

AFM were performed in air using a Nanoscope III (Digital Instruments, Santa Barbara, USA) operating in tapping mode. A 5 μl drop of diluted NPV suspension was placed on mica slide previously fixed on a metallic magnetic support by bi-adhesive tape. Sample was left to desiccate overnight in a desiccator. The analysis was performed on a 2 × 2 micron surface at a scanning rate around 3.2 Hz.

Cryo-TEM samples were prepared at ambient conditions. A 2-μl drop of solution was placed on a pure thin bar 600-mesh TEM grid (Science Services, Munich). The drop was blotted with filter paper until it was reduced to a thin film (10–200 nm) spanning the hexagonal holes of the TEM grid. The sample was then vitrified by rapidly immersing into liquid ethane near its freezing point. The vitrified specimen was transferred to a Zeiss EM922 transmission electron microscope for imaging using a cryoholder (CT3500, Gatan). The temperature of the sample was kept below −175 °C throughout the examination. Specimens were examined with doses of about 1000–2000 e/nm^2 at 200 kV. Images were recorded digitally by a CCD camera (Ultrascan 1000, Gatan) using an image processing system (GMS 1.4 software, Gatan).

2.7. Dimensional and ζ potential analysis

The determination size and surface charge of NPV was performed using a Zetasizer 3000 PCS (Malvern Instruments, Malvern, UK) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing it with detergent and rinsing it twice with water for injections. Measurements of size analysis were made at 25 °C at an angle of 90°. Data were interpreted using the Contin software.

2.8. Evaluation of K_D between NPV and DTK

The DTK-NPV affinities were evaluated calculating the dissociation constant (K_D) of their binding equilibrium, as follows. Aqueous solutions containing six increasing concentration of DTK, ranging from 0.06 to 2 mg/ml, were singularly incubated with a 10 mg/ml of empty NPV (PO/T, 1:1, w/w) suspension for 6 h at 180 rpm using a KS250 basic orbital stirrer (IKA Labortechnik, Staufen, Germany). The incubation volume was 300 μl. Afterwards, each sample was filtered through 10,000 NMWL pore filters (Micron YM-10, Millipore Corporation) and then analysed using the HPLC system described above, in order to evaluate the content of free peptide [P]. The concentrations of the peptide bound to the non-phospholipid vesicles [NP] were obtained by the difference of the total concentrations of peptide used for each sample with the corresponding [NP] values. The concentrations of free non-phospholipid vesicles [N] were obtained by the difference between their total concentration and the [NP] values. Dissociation constants were calculated as K_D = [N] [P]/[NP] for each concentration of DTK introduced in the system after 6 h of incubation. The mean ± S.D. values were obtained from the K_D values corresponding to the six different concentrations of DTK incubated.

2.9. In vitro studies: model drug release and stability

In vitro model drugs release profile was obtained by dialysis method. The release experiments were conducted at 37 °C. Typically, 2 ml of BPTI or TRYP (2 mg/ml) or 0.5 ml of DTK (1 mg/ml) containing NPV were placed into a dialysis tube (molecular weight cut off 10,000–12,000; Medi Cell International, UK), then placed into 30 ml for BPTI and TRYP or 10 ml for DTK of 0.1 M phosphate buffer, pH 7.4 under orbital stirring at 100/mot/min overnight. One hundred and fifty microlitres of sample were withdrawn at regular time intervals from 1 to 24 h and analysed by HPLC or UV/vis spectroscopy for DTK and BPTI or TRYP content.

Table 1
Composition of NPV produced

NPV name	Composition	Weight ratio of components	Final NPV concentration (mg/ml)
POS	Polyglycero-6-oleate/sorbitan monolaurate	1:1	10
POT	Polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate	1:1	10
POTC	Polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate / cholesterol	1:1:0.1	10.5

2.10. Cell growth studies

The possible toxic effect of empty NPV were determined on in vitro cultured human leukemic K562 cells (Lozzio and Lozzio, 1975) and murine leukemic L1210. Standard conditions for cell growth were alpha-medium (Gibco, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10% foetal calf serum (Irvine Scientific, Santa Ana, CA). Scalar concentrations of NPV were incubated with cells at 37 °C in 5% CO_2 and 90% humidity ambient for 5 days in a Sterilcult 200 incubator (Forma Scientific, USA). Cell growth was determined by counting with a Model ZF Coulter Counter (Coulter Electronics Inc., Hielah, FL). Counts of viable cells were performed after 0.1% Trypan blue exclusion test.

2.11. Data analysis and statistics

The data are expressed as mean ± S.D. Statistical analysis was performed by the Student's *t*-test or analysis of variance (ANOVA), followed by SPSS 11.5 for Windows. The level of significance was taken at $p < 0.05$.

3. Results and discussion

3.1. NPV preliminary study

As a preliminary study, the preparation of empty NPV was performed by testing different vesicle compositions, namely polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate (POT), polyglycero-6-oleate/polyoxyethylen sorbitan monolaurate/cholesterol (POTC) and polyglycero-6-oleate/sorbitan monolaurate (POS). The components were chosen in order to consider the effect on NPV of the presence of (a) polyoyethylene chain and (b) cholesterol. Compositions, weight ratios and final concentration of components are reported in Table 1.

NPV were prepared by direct hydration and sonication as described in Section 2.2. In particular, in order to allow the full hydration of the sample, a warm aqueous buffer was used to hydrate the non-ionic surfactant/lipid thin layer. In addition, on the basis of previous results (Cortesi et al., 2006), an overnight gentle shaking, at room temperature, was performed to get an encapsulation enhancement of the chosen model drug.

After preparation, NPV were then subjected to extrusion in order to obtain homogeneous vesicle size. Dimensions were measured by PCS analysis and the obtained results, before and after extrusion, were reported in Table 2 as the average of three independent determinations ± S.D. The reported results show that, as expected, vesicle size of all extruded vesicles preparations present a narrow size distribution with respect to the corresponding not extruded ones. NPV extruded through 200 nm pore size filters, are indeed characterized by polydispersity index comprised between 0.16 and 0.20; while those not extruded showed polydispersity index quite close to 1.00. In general, POS, POT and POTC suspensions are constituted of vesicles with an average diameter reflecting the pore size of the utilized membrane, namely 213.85 ± 0.35, 149.90 ± 2.20 and 153.95 ± 0.45 nm.

3.2. Preparation and characterization of drug containing NPV: trypsin

Since no remarkable problem was obtained by the three tested compositions of NPV, the ability to encapsulate TRYP as model drug was firstly investigated. Drug containing NPV were obtained as described for empty NPV. In order to reduce NPV dilution, un-encapsulated model drug was removed using the ultrafiltration method as described in Section 2.2. This technique allows the easy and rapid separation of the free drug without destabilizing the system. By this method, the encapsulation efficiency reached for TRYP was 49.37 ± 0.68, 59.31 ± 2.26 and 55.52 ± 1.39% for POS, POT and POTC NPV, respectively (see Table 3).

As reported in Table 3, it seems that, as determined by photon correlation spectroscopy (PCS), the presence of the TRYP induce a slight increase of NPV size with respect to the corresponding empty ones. POS, POT and POTC NPV are homogeneously polydispersed and characterized by size

Table 2
Size, polydispersity and ζ potential of empty NPV

NPV name	Before extrusion		After 200 nm pore size extrusion		ζ Potential[a]
	Diameter ± S.D. (nm)[a]	Polydispersity[a]	Diameter ± S.D. (nm)[a]	Polydispersity[a]	
POS	507.5 ± 23.7	1.00	213.8 ± 0.35	0.20 ± 0.04	−82.26 ± 4.65
POT	407.2 ± 19.6	1.00	149.9 ± 2.20	0.17 ± 0.02	−28.70 ± 3.70
POTC	274.3 ± 5.8	0.58 ± 0.07	153.9 ± 0.45	0.16 ± 0.05	−26.25 ± 4.51

[a] Data are the mean of three independent analyses ± S.D.; $p < 0.05$.

Table 3
Characteristics of NPV-containing trypsin

NPV name	ζ Potential	Before extrusion		After 200 nm pore size extrusion		Drug experiment	
		Diameter (nm)	Polydispersity	Diameter (nm)	Polydispersity	% Of drug encapsulated	Drug content (mg/ml)
POS	−56.4 ± 0.9	326.8 ± 7.2	0.88 ± 0.06	257.0 ± 4.9	0.57 ± 0.02	49.4 ± 0.7	1.31 ± 0.07
POT	−10.5 ± 0.6	260.3 ± 3.1	0.70 ± 0.07	146.4 ± 2.8	0.18 ± 0.03	59.3 ± 2.2	1.15 ± 0.13
POTC	−37.3 ± 0.1	–	–	163.0 ± 0.5	0.19 ± 0.08	55.5 ± 1.4	1.17 ± 0.05

Data are the mean of three independent analyses ± S.D.; $p < 0.05$.

of 257.00 ± 4.95, 146.40 ± 2.76 and 163.00 ±0.55 nm, respectively. On the other hand, the presence of cholesterol in the preparation induces an increase of the mean size both for sonicated and extruded NPV.

The dimensions of TRYP containing NPV were controlled over 6 months. In Fig. 1 the dimensional variations over time of empty (panel A) or TRYP-containing (panel B) NPV are reported. Both empty and drug-containing POT and POTC NPV proved to maintain their size over the considered 6 months. In particular, empty POTC-NPV are characterized by size around 155 nm, empty POT-NPV show dimensions around 150 nm and TRYP-containing POTC-NPV around 163 nm and finally TRYP-containing POT-NPV around 147 nm.

Concerning POS-NPV, the dimensions of empty vesicles are comprised between 213 and 223 nm while TRYP-containing vesicles are comprised between 257 and 325 nm. On the basis of this last higher dimensional range, POS-NPV is considered more unstable with respect to POT- and POTC-NPV.

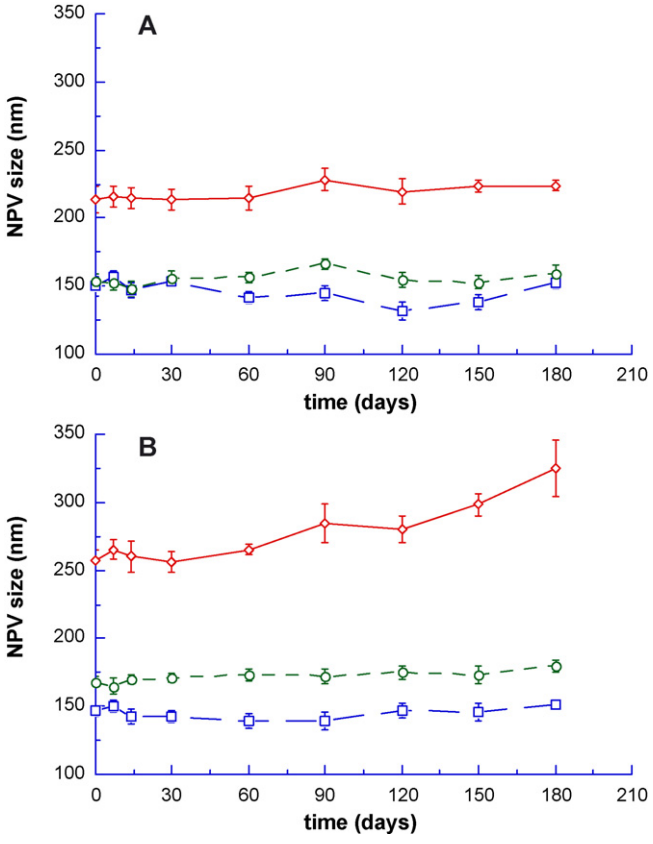

Fig. 1. Effect of ageing on dimensions of POS (◊), POT(□) and POTC (◯) NPV. Panel A: empty NPV. Panel B: NPV containing TRYP. Data represent the average of three independent experiments ± S.D. ($p < 0.05$).

3.3. Preparation and characterization of drug containing NPV: BPTI and DTK

On the basis of the above reported results, POT- and POTC-NPV were chosen to carry BPTI and DTK. All the preparations were obtained by hydration method as described in Section 2. In addition, the overnight gentle shaking, at room temperature, was performed to possibly obtain an encapsulation enhancement of the model drug.

It has to be underlined that, on the basis of previously published results on DTK containing liposomes (Cortesi et al., 2006), DTK-NPV were not subjected to extrusion in order to avoid peptide precipitation on the polycarbonate filter. The increase in homogeneity of NPV was obtained by sonicating the suspension for 4–5 min. In these conditions the stability of DTK was preserved as demonstrated by HPLC analysis (see Fig. 2).

Afterwards, ultrafiltration was performed to determine the encapsulation efficiency of both BPTI and DTK within NPV. The encapsulation efficiency reached in POT-NPV was 57.94 ± 1.63% for BPTI and 62.14 ± 1.32% for DTK; while in POTC-NPV was 62.37 ± 6.35 and 59.61 ±4.83% for BPTI and DTK, respectively (see Table 4). The obtained results are in agreement with respect to other vesicle-containing hydrophilic molecules characterized by percentages generally below 25–35% (Betageri et al., 1993).

From the analysis of the data reported in Tables 3 and 4 it is evident that all the prepared NPV are able to encapsulate more than 50% of the model drugs used in the present study. In particular, the lower percentage of encapsulation found for TRYP with respect to BPTI and DTK could possibly be ascribed to the lower temperature of hydration used during NPV preparation (i.e. 25 °C for TRYP and 40 °C for BPTI and DTK).

Fig. 3 depicts the Cryo-TEM images of POT-NPV empty (A) or DTK-containing (B). POT composition was taken as an example. In the case of empty-NPV many structures are evident, i.e. spherical vesicles, partially multilamellar, large lamellar structures with normally two layers and at least two fractions of small micelles between the vesicles.

The addition of small amounts of peptide induces a high change in the structure of NPV, such as the formation of

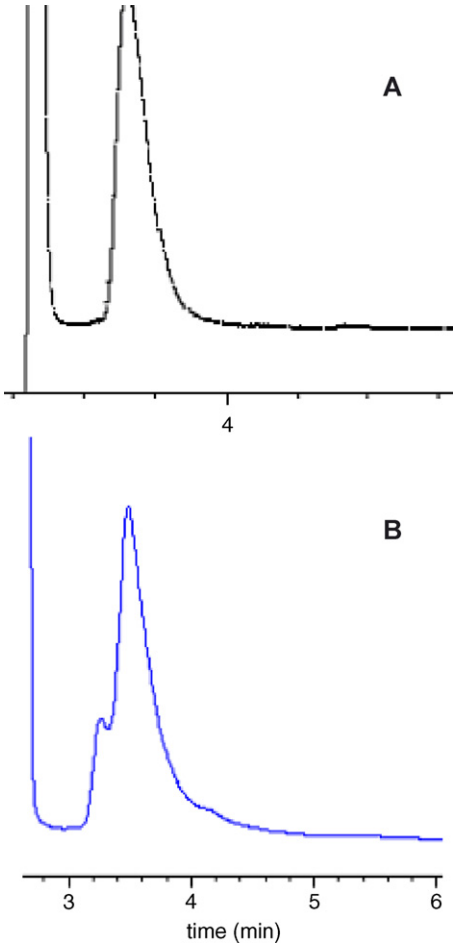

Fig. 2. HPLC chromatogram of DTK before NPV inclusion (panel A) and after NPV sonication (panel B).

membranous tubes and vesicular structures that interfuse and probably form the macroscopic deposits responsible for streaks.

However, DTK-NPV may show considerable amounts of drug in their external surface, allowing the peptide to be promptly available in the administration site. These conclusions can be ascribed to the analysis of the binding equilibrium between DTK and empty NPV. This analysis allowed us to find dissociation constants (K_D) in the high millimolar range. The mean K_D value obtained after 6 h of incubation, namely $3.8 \pm 0.4 \times 10^{-3}$ M (see Table 5), highlighted a weak interaction indeed between DTK and NPV.

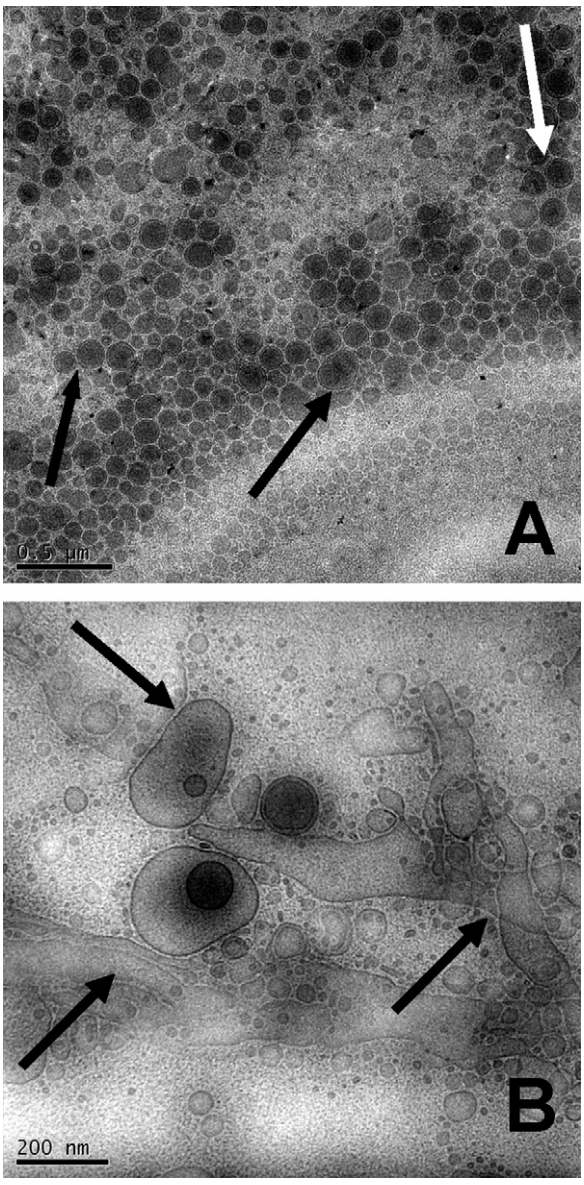

Fig. 3. Cryo-TEM microphotographs of NPV. Panel A: empty NPV. Arrows indicate spherical vesicles, partially multilamellar and large lamellar structures. Panel B: NPV containing DTK. Arrows indicate membranous tubes and vesicular structures. The bar is 500 and 200 nm in panels A and B, respectively. POT composition was taken as an example.

Table 4
Characteristics of BPTI- or DTK-containing NPV

NPV name	ζ Potential	Day 1		Drug experiment		Day 90	
		Diameter (nm)	Polydispersity	% Of drug encapsulated	Drug content (mg/ml)	Diameter (nm)	Polydispersity
BPTI							
POT	−13.05 ± 0.7	135.9 ± 4.2	0.32 ± 0.03	57.9 ± 1.6	1.98 ± 0.02	145.4 ± 3.7	0.22 ± 0.10
POTC	−16.71 ± 0.6	156.4 ± 0.8	0.13 ± 0.03	62.4 ± 6.3	1.62 ± 0.01	157.7 ± 0.8	0.45 ± 0.04
DTK							
POT	−35.15 ± 0.7	217.2 ± 3.4	0.65 ± 0.01	62.1 ± 1.3	1.09 ± 0.03	403.8 ± 27.4	0.79 ± 0.02
POTC	−22.31 ± 1.1	390.6 ± 8.7	0.95 ± 0.03	69.6 ± 4.8	0.99 ± 0.02	407.8 ± 55.3	1.00 ± 0.01

Data are the mean of three independent analyses ± S.D.; $p < 0.05$.

Table 5
Evaluation of K_D between NPV and DTK

[P]	[N]	[PN]	K_D	$K_D \pm$ S.D.[a]
2.70×10^{-4}	7.90×10^{-3}	5.30×10^{-4}	4.0602×10^{-3}	$3.8 \pm 0.4 \times 10^{-3}$
2.25×10^{-4}	8.03×10^{-3}	4.69×10^{-4}	3.8523×10^{-3}	
4.00×10^{-5}	8.40×10^{-3}	9.60×10^{-5}	3.5000×10^{-3}	
2.25×10^{-5}	8.43×10^{-3}	7.45×10^{-5}	2.5460×10^{-3}	
1.30×10^{-5}	8.47×10^{-3}	3.37×10^{-5}	3.2674×10^{-3}	
1.02×10^{-5}	8.49×10^{-3}	1.51×10^{-5}	5.7350×10^{-3}	

[a] Standard deviation; data are obtained as indicated in Section 2.

3.4. Size stability of NPV containing BPTI or DTK

The stability of encapsulated TRYP and BPTI molecules was evaluated by assaying their enzymatic activity. Particularly, both activity and enzyme concentration were measured by determining the hydrolysis rate of a synthetic substrate. It has to be underlined that this method not only allows a quantitative determination of the enzyme, but also demonstrate that after encapsulation the enzyme maintains its biological activity. Concerning DTK, its integrity was supported by HPLC analysis.

NPV containing BPTI or DTK were checked for dimensional stability over 3 months by PCS analysis. Particularly, BPTI containing NPV made of POT and POTC remain stable in size over the 3 months showing dimensions comprised between 156.40 and 157.70 nm for POTC and 135.88 and 145.45 nm for POT. On the other hand, DTK-containing NPV (i.e. POT vesicles) are characterized by mean size passing from 217.16 to 403.8 nm over 3 months. In Fig. 4 AFM images of POT-NPV 1 and 60 days old are reported. It has to be pointed out that POT-NPV pass from 113.28 to 183.59 nm within 2 months. These values are in agreement with PCS data confirming a quite good stability of vesicles.

Taken together the above reported results demonstrated that the aging of the NPV suspension does not heavily influence the initial vesicle size in the case of BPTI-containing POT and POTC (as confirmed by the polydispersity values, data not shown), while DTK-containing NPV showed a doubling of their size within 2 months.

3.5. In vitro studies: peptide release and stability

The study of the *in vitro* availability and drug release profiles from colloidal carriers (such as liposomes, micro- and nanoparticles) has commonly been performed by two different approaches

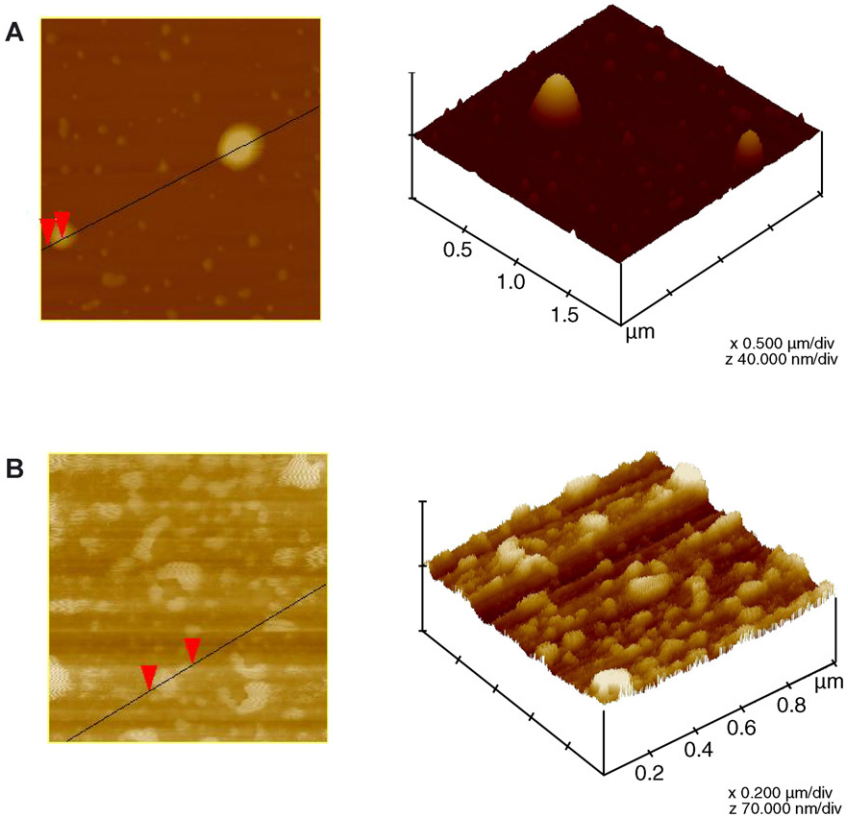

Fig. 4. AFM images of DTK-containing POT-NPV after 1 (panel A) and 60 days (panel B) from preparation. Each sample analysis was reported as bi- and three-dimensional image. Red arrows indicate 118 and 184 nm in panels A and B, respectively.

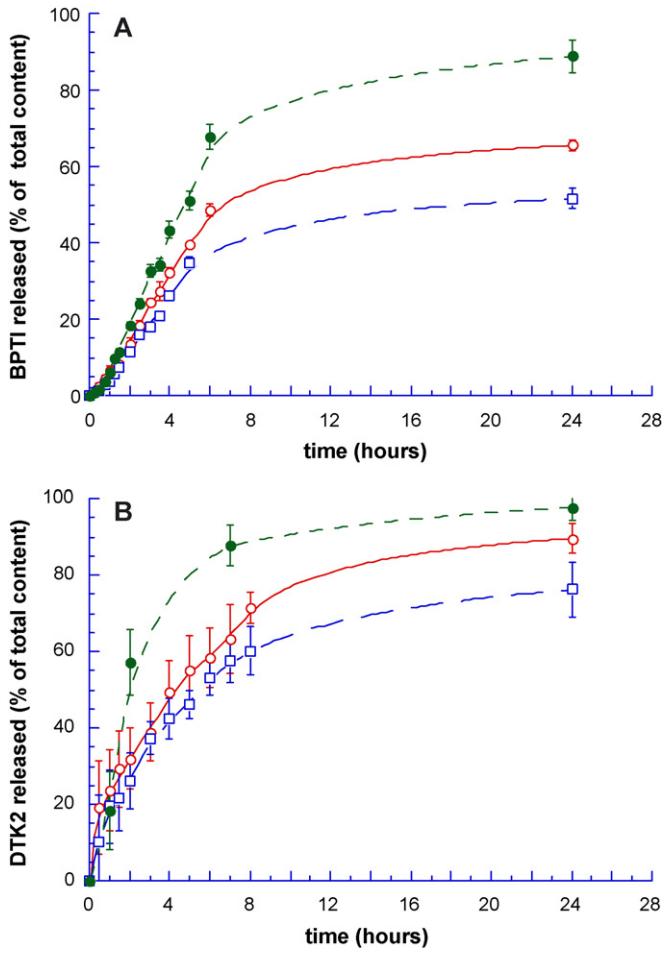

Fig. 5. Release profile of BPTI (panel A) and DTK (panel B) from POT (□) and POTC (◯) NPV. The releases were determined by dialysis method. As comparison drug release from aqueous solution is reported (●). Data represents the average of three independent experiments ± S.D. ($p < 0.05$).

(Magenheim and Benita, 1991), namely dialysis techniques and continuous flow methods. In the present investigation the horizontal shaker method was employed. It should be pointed out that the dialysis method was criticised (Washington, 1990; Nastruzzi et al., 1994) because of its low in vivo predictivity in the case of intravenous or oral administration of microparticles, where biological sink conditions are predominant.

Nevertheless, in our opinion, dialysis technique could more strictly reproduce the situation of subcutaneously injected or mucousal administered particles in which the carriers are presumably surrounded by a stagnant layer that, causing a slow diffusion of the drug, does not reproduce sink conditions.

In Fig. 5 the BPTI and DTK release profiles from NPV and, as a comparison, from aqueous solutions, determined by dialysis, are reported.

From the analysis of the reported release profiles it is evident that the free solution of both DTK and BPTI linearly increase with time until reaching plateau between 6th and 7th hour for BPTI, and between 8th and 9th hour for DTK. Concerning the 100% achievement of the aqueous curves, it should be underlined that DTK solution reaches the 100% after 24 h, taking into consideration the S.D. values. In addition, DTK integrity was supported by HPLC analysis. For BPTI, the not reached 100% release should be possibly ascribed to the method of quantification of the polypeptide. In fact, the concentration of BPTI molecule was evaluated by assaying its enzymatic activity. This method, surely useful to demonstrate the keeping of the biological activity of BPTI after encapsulation, only evaluates the active enzyme and not the un-active one. In this view, a possible underestimation of the enzyme concentration can be obtained.

Table 6
Antiproliferative activity of empty NPV

NPV name	IC50 (mM)	
	K562 cells	L1210 cells
POS	0.167 ± 0.004	0.200 ± 0.006
POT	0.076 ± 0.020	0.107 ± 0.018
POTC	0.217 ± 0.004	0.242 ± 0.002

Data are the mean of three independent analyses ± S.D.; $p < 0.05$.

The BPTI and DTK release profile from NPV is typically constituted of two different phases: an initial period, characterized by a relatively fast phase, followed by a second one, characterized by a slower release of the drug. In particular, when BPTI and DTK are NPV carried, the release is characterized by a more evident control with respect to those obtained by the corresponding free form in aqueous solution. However, it has to be underlined that in the case of BPTI release a slight lag phase is evident. Moreover, the plateau is reached between 6th and 9th hour showing a maximum around 50% for BPTI and around 60–70% for DTK. These data give rise to a low bioavailability that can be quantified, in the overall fold investigated, around 20–30%. This value seems to depend on the drug ability to bind the vesicles at the external surface, in agreement with the above-described results where, under similar experimental conditions, almost 25% of DTK is able to bind the empty NPV.

In addition, it should be stressed that both the release profiles are a function of NPV composition. The presence of cholesterol, indeed, by reducing membrane permeability and increasing bilayer rigidity, determine a reduction of both BPTI and DTK release (see Fig. 5).

3.6. *In vitro studies: NPV activity on cell growth*

In order to obtain information on possible toxicity of the obtained vesicles, in vitro antiproliferative activity of empty NPV was performed on both human erythroleukaemic K562 and murine lymphocyte L1210 leukaemic cells. For the experiments scalar concentrations of the single component were tested, namely 0.059–1.9 mM for POS NPV, 0.264–0.846 mM for POT NPV and 0.0716–2.3 mM for POTC NPV. After 6 days of cell culture, cells were electronically counted and the number of cells per millilitre was compared with the values obtained in the case of untreated cells. As clearly evident from the IC_{50} values reported in Table 6, all the NPV preparations are characterized by a discrete antiproliferative activity ($p < 0.05$). In addition it should be mentioned that K562 cells seem to be more responsive to NPV effect as compared to L1210 cell line.

4. Conclusions

In the last years, a large number of studies were performed on the possible use of liposomal and niosomal vesicles as therapeutic drug carrier systems aimed to the reduction of drug toxicity by alterating their pharmacokinetics or to the modification of the drug delivery in order to prolong the drug action at the target site. The present investigation led to a simple, but positive result in the pharmaceutical technology area. Particularly, our study indicates that the produced non-phospholipid vesicles containing three model molecules, such as TRYP, BPTI and DTK (i) are able to encapsulate the model drugs over 49%, (ii) are characterized by dimensions compatible with applications on the mucous membrane, (iii) remain stable in size for at least 3 months and (iv) can release the peptide following the same behaviour of the corresponding free solution.

It has to be underlined that the results on the investigations of the physicochemical interactions between NPV and the model drugs used in the present study will be the object of a future paper (Cortesi et al., manuscript in preparation). Moreover, further studies are in progress in order to evaluate the ability of the different NPV to interact to the cells at the mucous membrane site and efficiently release the encapsulated peptide.

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