

LIPOSOMAL NANODELIVERY SYSTEM FOR PROTEASOME INHIBITOR ANTICANCER DRUG BORTEZOMIB

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Abstract

Bortezomib is the first therapeutic agent designed to inhibit mainly the activity of the 26S proteasome. It is used for the treatment of multiple myeloma. The objective of this study was to develop and optimize liposomal formulation of bortezomib in order to improve the encapsulation efficiency. For evaluating the encapsulation efficiency it was investigated the effect of two parameters: the drug/lipid molar ratio and the pH of the medium. The liposomal formulation of bortezomib was obtained by mixing a solution of bortezomib with SUV liposomes (small unilamellar vesicle). A transmembrane pH gradient was obtained by creating a difference of pH between internal medium of the liposomes and the external medium in which the liposomes were suspended. When preparing the liposomes by using a pH gradient it was obtained an encapsulation efficiency of 48 % for the molar ratio bortezomib:lipid 1:5.

Rezumat

Bortezomib este primul agent terapeutic conceput pentru a inhiba în principal activitatea proteazomului 26S. Acesta este utilizat pentru tratamentul mielomului multiplu. Obiectivul acestui studiu a fost de a dezvolta și optimiza formularea lipozomală a medicamentului bortezomib, pentru a îmbunătăți eficiența de încapsulare. Pentru a evalua eficiența încapsulării a fost investigat efectul a doi parametri: raportul molar medicament/lipide și pH-ul mediului. Formularea lipozomală a fost obținută prin amestecarea unei soluții de bortezomib cu lipozomi SUV liposomes (*small unilamellaar vesicle*). Un gradient de pH transmembranar a fost obținut prin crearea unei diferențe de pH între mediul intern al lipozomilor și mediul extern în care aceștia au fost suspendați. La pregătirea lipozomilor utilizând un gradient de pH, s-a obținut un randament de încapsulare de 48% pentru raportul molar bortezomib:lipide 1:5.

Keywords: liposomes, bortezomib, drug encapsulation, thin-film hydration method, encapsulation efficiency, Stern-Volmer constant

Introduction

Along with the cardiovascular diseases, cancer continues to be one of the most serious diseases that humanity is facing. This disease has a mechanism of anarchic and uncontrolled cell proliferation. Modern drugs used in oncology are able to destroy tumours successfully, enabling quick recovery if the patient is diagnosed in early stage. Bortezomib (also named PS-341 or Velcade) belongs to this class of drugs. It generated promising preclinical and clinical results for treatment of multiple melanomas. Bortezomib is a boron acid derivative presenting anti-tumour effects by inhibiting particularly the proteasome 26S [8]. The approval of this anticancer agent by the U.S. Food and Drug Administration and the European Agency for the Evaluation of Medicinal Products for the treatment

of multiple myeloma led to its recognition as the first drug in the class of proteasome inhibitors [6, 11].

Furthermore, the antitumor activity of Bortezomib was demonstrated against a large variety of tumours types, including malignant tumours of the central nervous system [9], melanoma, lung cancer [2], colon cancer, ovarian cancer, kidney cancer and prostate cancer [16]. Preclinical antitumor activity of proteasome inhibitor was observed for leukaemia [5] and lymphoma [19]. The inhibition process of tumour cell growth due to bortezomib cytotoxic activity was found to be unique compared to other anti-cancer agents, suggesting a new cytotoxic mechanism of inhibition [3]. However, bortezomib has some side effects such as gastrointestinal symptoms, thrombocytopenia, neuropathic pain,

anemia, lymphopenia and peripheral neuropathy [15].

Recently, Ronggui Hu [17] presented encapsulation of bortezomib into hollow mesoporous silica nanospheres for improving the efficacy for non-small cell lung cancer therapy. The results demonstrated that bortezomib loaded nanostructures showed improved tumour suppressing effect compared to free bortezomib.

Other carrier vectors frequently used are the liposomal systems [1]. Applications of the liposomes as controlled release systems of drugs increased due to their ability to incorporate and protect many types of therapeutic biomolecules both in the lipid bilayer and in the internal aqueous environment [13]. Liposomes are used in treatment programs because of their capacity to reduce the toxic effects of the drugs, to increase the circulation time [14, 22, 22] and effectiveness of the agents [7] and due to their biodegradable properties [4].

The main objective of this study was to quantify the encapsulation process of the anticancer agent bortezomib into liposomes, by evaluating the encapsulating efficiency and by studying the interaction between the polypeptide bortezomib and the DPPC lipids. It was investigated the effect of two parameters (the drug: lipid molar ratio and the pH of the medium) on the encapsulation efficiency.

Materials and Methods

Chemicals and Reagents. The reagents 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Avanti Polar) and bortezomib (Millennium Pharmaceuticals) were used without any further purification. For fluorescence measurements, the liposomes were marked with the fluorophore 1,6 – diphenyl – 1,3,5 – hexatriene (DPH), the molar ratio lipid / fluorophore was 500/1.

Acquisition and analysis of data. The spectrofluorimetric experiments were carried out

using a Perkin Elmer LS 55 Fluorescence Spectrometer with FLWinLab Soft. The results were processed with Microcal Origin Programme (version 6.0). The emission spectra were recorded at the bortezomib excitation wavelength ($\lambda_{ex} = 265$ nm) and at the DPH excitation wavelength ($\lambda_{ex} = 356$ nm). All experiments were performed at 47°C.

Preparation of liposomes. The liposomes were prepared using the thin-film hydration technique [12], appropriately adapted for our purposes. For the hydration of the lipid film, a phosphate buffer solution was used, pH 7.2, 60 mM concentration. The DPPC phospholipids (0.3 mM as final concentration in liposome dispersion) were dissolved in methanol. The solvent was evaporated using a Heidolph rotary evaporator. The lipid film obtained was hydrated with the phosphate buffer solution and vigorously shaken. Unilamellar liposomes were obtained by sonication for 30 minutes, with 30 % amplitude, using a Vibra-Cell VCX 750 Sonicator. Finally, the dispersion was centrifuged for 30 minutes at 12 000 rpm. The liposomal formulation of drug was obtained by mixing a solution of bortezomib with liposomes. The molar ratio between drug and DPPC lipid was 1:15 (sample 1), 1:10 (sample 2), 1:7 (sample 3) and 1:5 (sample 4). The samples were incubated at 47°C for 8 hours.

To optimize the encapsulation efficiency it was prepared a liposome dispersion by creating a transmembrane pH gradient, with a difference of pH between liposomes internal medium (pH 8.2) and the external medium (pH 6) in which they were suspended.

Drug Loading. The encapsulation of drug molecules in DPPC liposomes was achieved by gently stirring a solution of bortezomib with the liposomes dispersion. The encapsulation efficiency was calculated using the following equation [18].

$$\% \text{ Encapsulation Efficiency} = \frac{\text{Encapsulated Drug}}{\text{Total Drug}} \times 100 \quad (1)$$

Moreover, based on the fluorescence signal modifications of the DPH fluorophore can be obtained information regarding the location of the bortezomib molecules into the liposomes. For calculating the bimolecular quenching constant of the DPH fluorescence, the Stern-Volmer equation [10] was used:

$$\frac{I_0}{I} = 1 + K_q \tau_0 [B] = 1 + K_{SV} [B] \quad (2)$$

where, I_0 and I represent the intensities of the fluorescence in the absence and respectively in the presence of the quencher (bortezomib), K_{SV} represents the Stern – Volmer constant, K_q – the

bimolecular quenching constant of the fluorescence, τ_0 is the lifetime of the DPH fluorescence in the absence of bortezomib, and $[B]$ represents the concentration of bortezomib. The bimolecular quenching constant of the fluorescence is given by the relation:

$$K_{SV} = K_q \tau_0 \quad (3).$$

The fluorescence method was used both to put into evidence the interaction between lipids and bortezomib and to estimate the encapsulation efficiency. When dissolved in aqueous medium, bortezomib presents a weak fluorescence emission at $\lambda_{em} = 420$ nm after excitation at a wavelength λ_{ex}

= 265 nm, characteristic for boron acid (Figure 1). All experiments were run in triplicate.

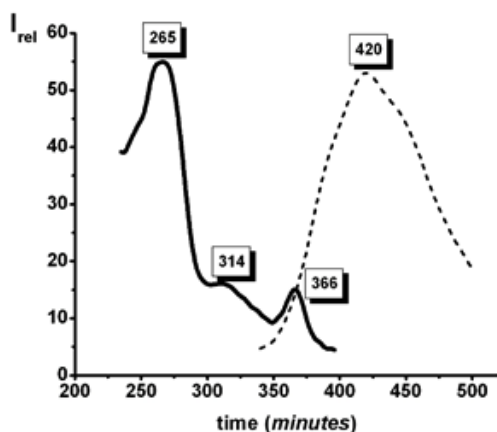


Figure 1.

Bortezomib excitation spectra (—) and emission spectra (---)

Results and Discussion

The study aimed to quantify and optimize the encapsulation process of the anticancer agent bortezomib in carrier vectors - liposomes. The efficiency of encapsulation in liposomes is given by the ability to prepare these vesicles for a higher percentage incorporation and retention of the drug molecules for a longer period of time.

Monitoring bortezomib fluorescence allowed the investigation of the influence of the molar ratio lipid:drug on the encapsulation process. The maximum of the fluorescence intensities were plotted as a function of the incubation time of bortezomib polypeptide with the dispersion of liposomes (Figure 2).

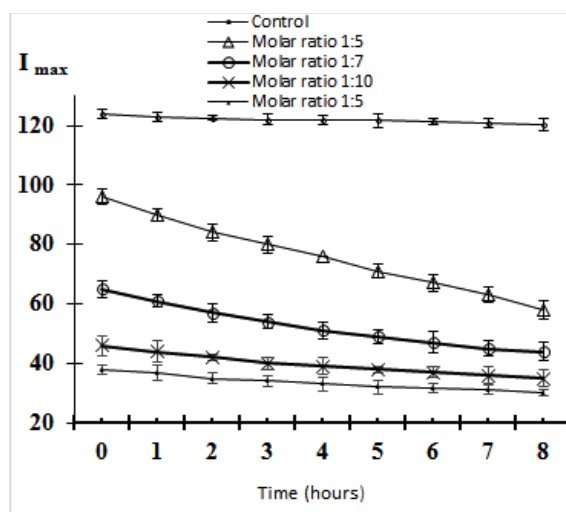


Figure 2.

Evolution of the maximum intensity recorded at λ_{em} bortezomib

It was observed a decrease in time of the relative intensity of the bortezomib fluorescence at 420 nm maximum emission according to the molar ratios bortezomib: DPPC. It can be considered that this decrease is due to the fact that the bortezomib encapsulated in the liposomes does not show the same fluorescence characteristics as when it is dissolved in solution. As the time of incubation increased, the concentration of bortezomib in solution and the intensity of the fluorescence emission decreased.

ΔI was determined by calculating the difference between the value of the bortezomib emission intensity at the initial moment and the values of the intensity recorded at the time τ . It was considered that this difference is due to the encapsulation of the bortezomib in the liposomes and it was used to estimate the amount of drug encapsulated. The linear regression equation was used and the corresponding concentration ΔI was considered as representing the total quantity of encapsulated drug, at that moment.

The efficiency of encapsulation was determined by using Equation 1 for the four samples (Figure 3). The calculation of the efficiency was performed separately for each hour of incubation, namely the total amount of drug used in calculation being represented by the amount of bortezomib left in the solution at the given moment. It can be noted that the encapsulation efficiency decreases from one hour to another, for each of the four samples. The efficiency of drug encapsulation decreases when the difference in concentration decreases.

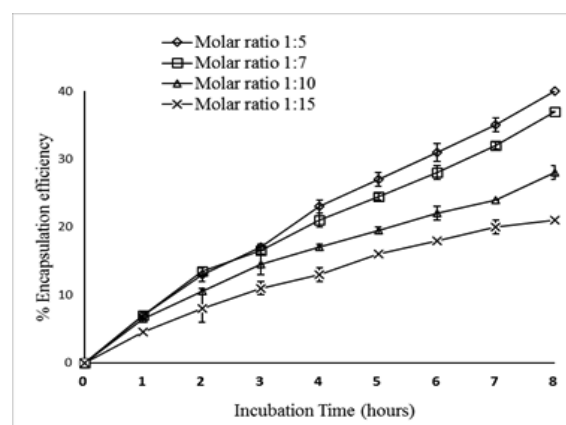


Figure 3.

The efficiency of encapsulation recorded at λ_{em} bortezomib

The final efficiency of bortezomib encapsulation was calculated using Equation 1. A maximum efficiency of encapsulation was obtained for sample 1 (approximately 40%). For sample 2 it was 37 %, for sample 3, 28%, and sample 4, 21%. This showed that the molar ratios drug:lipid with lower

values were favourable to bortezomib encapsulation in DPPC liposomes. The existence of a high gradient of bortezomib concentration, between the outside and inside of the lipid bilayer, favours a more efficient encapsulation.

In Figure 4 is presented the evolution in time of the maximum fluorescence intensity of the DPH fluorophore labelling the DPPC liposomes. An important feature of this fluorophore is that it only fluoresces when it interacts with lipids [10]. After about 30 minutes from the incubation of the liposomes with DPH, the fluorescence emission intensity of DPH at the maximum from 430 nm remains stationary. Moreover, for 8 hours were not recorded major changes of the DPH fluorescence emission, so the results are not influenced by its possible degradation.

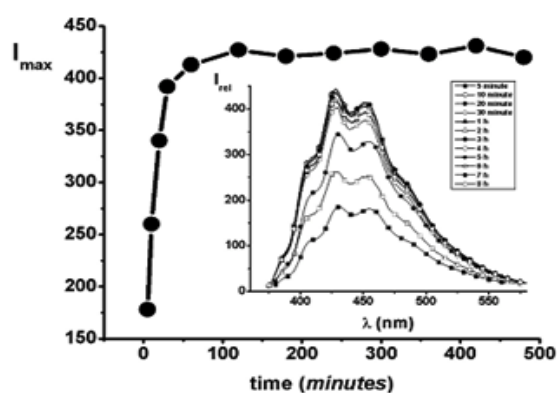


Figure 4.

The evolution in time of the maximum fluorescence intensity of DPH

The maximum intensities of the fluorescence were measured after recording the emission spectra by using the excitation wavelength of the DPH fluorophore. These were represented graphically as a function of the incubation time of the bortezomib polypeptide with the liposomes dispersion for each molar ratio bortezomib:lipid (Figure 5).

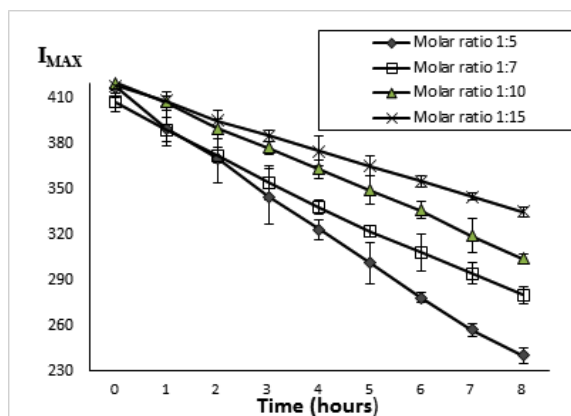


Figure 5.

The relative intensity of the DPH fluorescence emission as a function of the incubation time

The intensity of the DPH fluorescence emission, in the presence of the bortezomib, decreases in time. It can be concluded that the polypeptide acts as a quencher of the DPH fluorescence in the liposomes, the quenching respecting equation 2 [10].

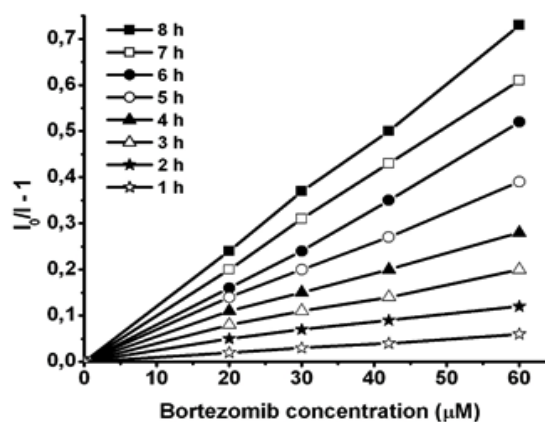


Figure 6.

Stern-Volmer equation for DPPC liposomes

In Figure 6 the Stern – Volmer equation is represented graphically for the DPPC liposomes labelled with the DPH fluorophore, in the presence of bortezomib at various concentrations.

Following the graphical representation of the Stern – Volmer equation a linear dependence was obtained between the ratio of the fluorescence intensity values, in the absence and respectively in the presence of the quencher, and the concentration of bortezomib. This linearity indicates that the DPH fluorophore is accessible to the quencher – bortezomib. As the DPH is inserted in the lipid bilayer, it means that the bortezomib penetrates the lipid bilayer, and thus, it can be encapsulated in the liposomes. By using the values of the slopes (Figure 6), the value of the Stern–Volmer constant K_{SV} , (Table I) was calculated for each incubation time of the liposomes with the bortezomib polypeptide. The value of the bimolecular quenching constant of the fluorescence K_x was calculated using equation 3, considering the value 10 ns for the lifetime of the DPH fluorescence without bortezomib (Table I).

Table I

The Stern-Volmer constant (K_{SV}) and the bimolecular quenching constant of the fluorescence (K_q) for the liposomes

Time (hours)	$K_{SV} (X 10^4 M^{-1})$	$K_Q (X 10^{12} M^{-1}S^{-1})$
1	0.09	0.09
2	0.19	0.19
3	0.32	0.32
4	0.46	0.46
5	0.64	0.64
6	0.92	0.92
7	1.02	1.02
8	1.21	1.21

According to the literature, the dynamic quenching due to the collision processes is characterized by a value of the bimolecular quenching constant of the fluorescence in the domain $10^{10} \text{ M}^{-1}\text{s}^{-1}$. When the values of this constant are somewhat higher, approximately $10^{12} \text{ M}^{-1}\text{s}^{-1}$, the quenching of the fluorescence occurs as a result of the formation of a complex between the fluorophore and the quencher – static quenching [10].

The values obtained for the bimolecular quenching constant of fluorescence for the DPPC liposomes labeled with DPH fluorophore vary in the range $(9 \div 64) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ for 1-5 hours and in the range $(0.92 \div 1.21) \times 10^{12} \text{ M}^{-1}\text{s}^{-1}$ for 6-8 hours. Consequently:

- within 1 – 5 hours of incubation of the bortezomib solution with SUV liposomes, the quenching of DPH fluorescence is due to some processes of collision between fluorophore and bortezomib, namely a dynamic quenching of the fluorescence;
- beginning with the sixth hour of incubation, the bimolecular quenching constants of the DPH fluorescence have values of 10, 15 times higher. This can be explained either by the formation of a non-fluorescent complex between DPH fluorophore and bortezomib in its fundamental state (static quenching), or by the existence of an effective sphere of the fluorescence quenching next to the linear molecule of the DPH [21].

The first version seems less probable, taking into account the redox proprieties of the bortezomib, and the encapsulation efficiency results, mentioned before. It is less probable that the bortezomib stands still in the hydrophobe area of the lipid bilayer and forms a complex with the DPH. It is more probable that the encapsulation due to the concentration gradient, should lead to an „agglomeration” when passing through the bilayer, especially in the area where the DPH has been inserted.

Considering the results of the electrochemical investigation of the bortezomib under pH variation, it was tested the encapsulation of the drug in liposomes prepared by creating a pH gradient. The study was carried out using sample 1 for which the best efficiency of encapsulation was obtained earlier.

It is interesting to note a significant increase of the drug accumulation in the carriers, as a response to the pH gradient. As it can be seen in Figure 7, the drug was accumulated much faster in the liposomes. The encapsulation efficiency was 48 % after 8 hours of incubation (Figure 8) which is 8 % higher than in the case of preparing liposomes by the conventional method. The t test results showed

that there is a significant difference between the two methods ($p = 0.004$).

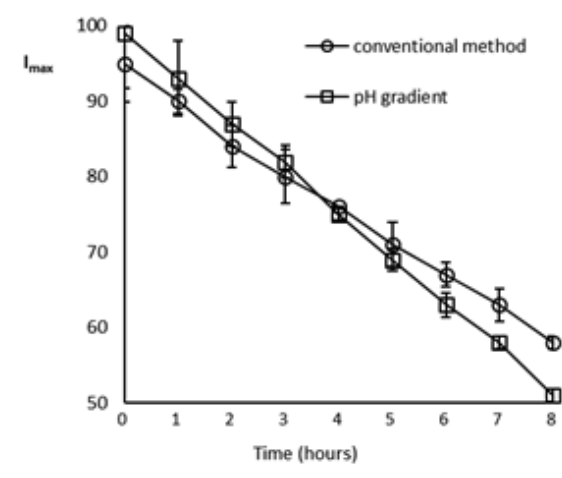


Figure 7.

The maximum intensity of the DPH fluorescence emission as a function of the incubation time

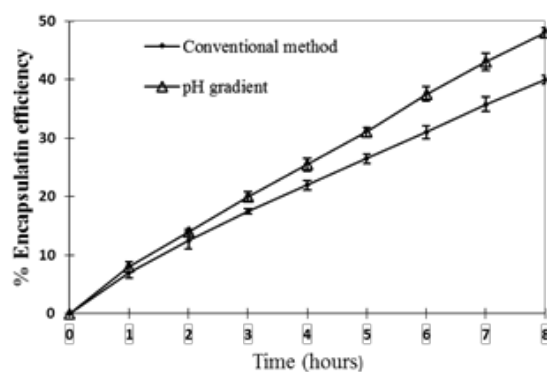


Figure 8.

Comparison between the encapsulation efficiency of bortezomib in liposomes

The fact that the pH gradient accelerates the decrease of the bortezomib fluorescence in solution represents an additional argument for the bortezomib encapsulation within the liposomes by a process of guided passing through the hydrophobic space of the lipid bilayer.

Conclusions

The existence of a concentration gradient of the bortezomib between the outside and the inside of the lipid bilayer, favours a more efficient encapsulation of the drug into the liposomes. In the case of liposomes preparation by using a pH gradient it was obtained an encapsulation efficiency of 48 % for the molar ratio bortezomib:lipid 1:5. The values calculated for bimolecular quenching constant of the fluorescence showed that the bortezomib encapsulation into liposomes, due to the gradient concentration, lead to an „agglomeration”

when passing through the lipid bilayer, especially in the area where the DPH has been inserted.

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