Colloids and Surfaces B: Biointerfaces 121 (2014) 1–10

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

The critical role of didodecyldimethylammonium bromide on physico-chemical, technological and biological properties of NLC

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ARTICLE INFO

Article history:
Received 11 March 2014
Received in revised form 8 May 2014
Accepted 15 May 2014
Available online 27 May 2014

Keywords:
Lipid nanoparticles
Factorial design
Turbiscan AG Station
Cryo-TEM
X-ray diffraction
Cell cultures

ABSTRACT

Exploiting the experimental factorial design and the potentiality of Turbiscan AG Station, we developed and characterized unmodified and DDAB-coated NLC prepared by a low energy organic solvent free phase inversion temperature technique. A 22 full factorial experimental design was developed in order to study the effects of two independent variables (DDAB and ferulic acid) and their interaction on mean particle size and zeta potential values. The factorial planning was validated by ANOVA analysis; the correspondence between the predicted values of size and zeta and those measured experimentally confirmed the validity of the design and the equation applied for its resolution. The DDAB-coated NLC were significantly affected in their physico-chemical properties by the presence of DDAB, as showed by the results of the experimental design. The coated NLC showed higher physical stability with no particles aggregation compared to the unmodified NLC, as demonstrated by Turbiscan® AGS measurements. X-ray diffraction, Raman spectroscopy and Cryo-TEM images allowed us to assert that DDAB plays a critical role in increasing the lipids structural order with a consequent enhancement of the NLC physical stability. Furthermore, the results of the *in vitro* biological studies allow the revisiting of the role of DDAB to the benefit of glioblastoma treatment, due to its efficacy in increasing the NLC uptake and reducing the viability of human glioblastoma cancer cells (U87MG).

1. Introduction

The optimization of formulation parameters together with the evaluation of the stability of a colloidal system are great scientific challenges of prime practical importance for the development of an effective formulation. The experimental factorial design has been achieving rising attention due to the avoidance of disadvantages of the traditional one-factor-at-a-time method to experimentation that is time and costs consuming [1]. The experimental design has been successfully used in previous works highlighting the possibility to collect a great amount of information, not only on the effect of a single variable but also on the interaction of different factors, thus reducing the numbers of the experiments [2,3]. Particle size (Zave) and zeta potential (ZP) are the most important parameters to evaluate the stability of a nanoparticulate system [2,4]. Stability studies are usually performed using the traditional visual observation, which is time-consuming and, sometimes, non-realistic. In order to overcome these limits we exploited the Turbiscan technology. The main advantage of Turbiscan is the ability to detect destabilization phenomena much earlier than the naked eye's operator, enabling faster and more relevant characterization (objective and measurable results) of suspensions [3,5]. Nanostructured lipid carriers (NLC) have been recently proposed for the systemic administration of lipophilic compound for the treatment of different brain diseases due to their extremely reduced mean size. In particular, lipid nanoparticles have been studied for the treatment of glioblastoma, exploiting the potentiality of anti-inflammatory drugs such

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http://dx.doi.org/10.1016/j.colsurfb.2014.05.024
0927-7765/

as curcumin and ferulic acid (FA) [6,7]. The interest in lipid nanoparticulate systems demonstrated by the research institutions and the pharmaceutical companies is confirmed by the various patents registered as regards the methods of preparation of lipid nanoparticles and their potential wide range of applications [8,9]. On the basis of these considerations, the aim of this work was the optimization of NLC as a new approach in glioblastoma treatment, using a coating layer of the cationic lipid didodecyldimethylammonium bromide (DDAB), which has been demonstrated to induce cell death in various types of tumor cell lines activating the caspase-dependent apoptosis pathway [10]. The effect of the addition of the drug (FA) and the cationic lipid on mean size, zeta potential and physical stability was evaluated using PCS and the recently developed Turbiscan® AG Station, in order to select the most promising system for the treatment of glioblastoma. These tools, in combination with the experimental factorial design, allowed us to achieve a great amount of direct, objective and measurable information on the NLC behavior, thus reducing both time and costs of the experimentation. X-ray diffraction, Raman spectroscopy, Cryo-TEM and cytotoxicity studies (MTT assay) on human glioblastoma cancer cell lines (U87MG) have also been performed on the unloaded NLC to evaluate the influence of the addition of DDAB on the lipid structure and NLC efficacy. *In vitro* biological studies (cytotoxicity and uptake evaluation by MTT and CLMS, respectively) have been carried out on FA-loaded unmodified and DDAB-coated NLC to assess the influence of the coating layer of DDAB on NLC effectiveness and ability to interact with U87MG cells.

2. Materials and methods

2.1. Materials

Cetyl palmitate (Cutina CP) was a gift from BASF Italia S.p.A. (Cesano Maderno (MB), Italy). Gliceryl Monooleate (Tegin O), Oleth-20 (Brij 98) and Isopropyl stearate (IPS) were purchased from A.C.E.F. S.p.a. (Piacenza, Italy). 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and didodecyldimethylammonium bromide (DDAB) were bought from Sigma–Aldrich (Milan, Italy). Ferulic acid (FA) was purchased from Fluka (Milan, Italy). Regenerated cellulose membranes (Spectra/Por CE; Mol. Wet. Cut off 3000) were supplied by Spectrum (Los Angeles, CA). Methanol, acetic acid and water used in the HPLC procedures were of LC grade and were bought from Merck (Milan, Italy). All other reagents were of analytical grade. U87MG human glioblastoma cancer cell lines were purchased from Cell Bank Interlab Cell Line Collection (Genova, Italy). Modified Eagle Medium (MEM) with 2 mM GlutaMAX, Heath Inactivated Fetal Bovine Serum (FBS, GIBCO), Normal Goat Serum (NGS, GIBCO), Sodium Pyruvate, Streptomycin and penicillin antibiotics, 0.05% Trypsin–EDTA solution, Phosphate Buffer Saline solution (PBS) were from Invitrogen (Milano, Italy).

2.2. Preparation of NLC

On the basis of previous study, Brij 98 (8.7%, w/w) and glyceryl monooleate (4.4%, w/w) were selected as surfactants in combination with cetyl palmitate (4%, w/w) and IPS (1%, w/w) as lipid mixture [7]. A low energy organic solvent-free phase inversion process (PIT method) has been used for the preparation of the NLC: briefly, the aqueous phase and the oil phase were separately heated at ~90 °C; then the aqueous phase was added drop by drop, at constant temperature and under agitation, to the oil phase. The mixture was cooled to 60 °C, successively subjected to three thermal cycling (90–60 °C) and then cooled to room temperature under slow and continuous stirring.

2.3. Full factorial design

An experimental design with a two/levels two variables, 2^2 full factorial planning, was performed for the optimization of the NLC. The regression analysis allowed obtaining the Eq. (1) as mathematical model:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \varepsilon \quad (1)$$

Eq. (1) was applied on two responses, particles size and zeta potential, to describe the principal effects and interaction among the identified variables, x_1 and x_2. Coded (−1; +1) levels were used for each independent variable: the −1 level corresponds to the lower value of each variable and +1 to the upper one. These limits were selected on the basis of previous studies and the optimization procedure was carried out within these domains. Concerning the Eq. (1): Y is the dependent variable or response, β_1 and β_2 are the coefficients of the respective independent variable, β_0 is the arithmetic mean response, β_{12} is the interaction term and ε the error term. Student's t-test and ANOVA were applied to verify the fitted model. Statistical analysis was considered significant when the p values were less than 0.05.

2.4. Photon correlation spectroscopy (PCS)

The particle size was determined by photon correlation spectroscopy (PCS) which yields the mean particle size (ZAve) and the polidispersity index (PDI), that provides the width of the particle sizes distribution. PCS was performed using a Zetasizer Nano S90 (Malvern Instruments, Malvern, UK) at a detection angle of 90°, at 25 °C, with a 4 mW He–Ne laser operating at 633 nm. Each value was measured in triplicate. The results are shown as mean ± standard deviation. The zeta potential values (ZP), which reflects the electric charge on the particle surface, were determined using the same equipment described previously at 25 °C. For the measurements, samples were diluted appropriately with ultrapurified water.

2.5. Stability studies

Samples were stored in dark vials and kept at three different temperatures (4, 25 and 40 °C) for 6 months. At different time intervals (2 weeks, 1, 2, 3, 4, 5 and 6 months) each formulation was analyzed by PCS to evaluate the variation of mean particles size, polidispersity index and zeta potential.

2.6. Turbiscan® AG Station

In addition to the stability followed by PCS, we also assessed the physical stability of the samples using an optical analyzer Turbiscan® AGS (Formulation, L'Union, France). The apparatus consists in the Turbiscan Lab Expert that is based on the analysis of the multiple dispersion of the light by concentrated suspensions, provided with an Aging Station, constituted by a robot with three thermo regulated blocks for the storage of the samples. In our experiments, 20 ml of each unloaded sample were placed in a cylindrical glass cell and positioned in the Turbiscan at three different storage temperatures: 25, 40 or 60 °C. The detection head was composed of a pulsed near-infrared light source (λ = 850 nm) and two synchronous transmission (T) and back scattering (BS) detectors. The T detector receives the light, which crosses the sample (at 180° from the incident beam), while the BS detector receives the light scattered backwards by the sample (at 45° from the incident beam). The detection head scanned the entire height of the sample cell (65 mm longitude), acquiring T and BS each 40 μm (1625 acquisitions in each scan). The measuring principle is based on the variation of the particle volume fraction (migration) or diameter (coalescence), resulting in a variation of BS and T signals [3,5]. The