Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 16–22

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Characterization of a liposome-based formulation of oxaliplatin using capillary electrophoresis: Encapsulation and leakage

Ulrik Franzen[a], Tam T.T.N. Nguyen[a], Charlotte Vermehren[b,1], Bente Gammelgaard[a], Jesper Østergaard[a,*]

[a] Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
[b] LiPlasome Pharma A/S, Lyngby, Denmark

ARTICLE INFO

Article history:
Received 4 November 2010
Received in revised form
20 December 2010
Accepted 26 December 2010
Available online 13 January 2011

Keywords:
Capillary electrophoresis
ICP-MS
Oxaliplatin
PEGylated liposomes

ABSTRACT

A capillary electrophoresis-based method to characterize a PEGylated liposomal drug formulation of the anti-cancer agent oxaliplatin was developed. Pharmaceutical characterization in terms of determination of the free and total oxaliplatin concentrations in the liposomal formulation was successfully performed allowing calculation of the percentage of encapsulated drug and encapsulation efficiency. The trapping efficiency was likewise calculated. The capillary electrophoresis method allowed liposome characterization in the intended formulation media (sucrose solution with low electrolyte concentration), and the attained results were consistent with inductively coupled plasma mass spectrometry measurements. Accelerated drug leakage studies were initiated by the sonication of the PEGylated formulation, using an ultrasound probe, subsequently the drug leakage was determined by capillary electrophoresis. The results obtained with the PEGylated liposomes demonstrate that capillary electrophoresis may be a useful tool for the characterization of liposomal drug formulations.

1. Introduction

Oxaliplatin is an anti-cancer agent used for the treatment of advanced colorectal cancer and was first marketed under the name Eloxatin® by Sanofi-Aventis [1]. Oxaliplatin (Fig. 1) belongs to the third generation of anti-cancer platinum compounds and shows a better safety profile than cisplatin (first generation) and carboplatin (second generation) [2]. However, the use of oxaliplatin is still associated with a range of side-effects. In order to improve the efficacy and reduce the side effects of anti-cancer drugs (including platinum-based), different approaches using nanoparticulate delivery systems have been taken including liposomes, albumin-based particles, dendrimers, different inorganic particles, and polymeric micelles [3–5].

Nanoparticulate systems in form of liposomes have been studied extensively, which have resulted in several marketed liposomal products [6–9]. Main advantages associated with the encapsulation of a drug substance within a liposomal drug delivery vehicle include the possibility of decreasing the toxicity of the dosed drug, prolongation of the plasma half-life of the drug, targeting to specific molecules or structures in the body or having a triggered drug release induced by acid, light, heat or enzymes [8–10].

The application of nanoparticles as drug delivery systems requires a thorough characterization of the delivery system in order to ensure a reproducible effect of the drug. A model drug formulation of the anti-cancer agent oxaliplatin, based on PEGylated liposomes where polyethylene glycol (PEG) has been covalently attached to one of the constituting phospholipids (Fig. 1), was investigated in this study. Such PEGylated liposomes have been widely used in order to increase plasma half-lives of drugs by preventing the liposomes from being taken up by the reticuloendothelial system (RES) [8,9,11,12]. The efficacy of the liposomal drug formulation subject to study is based on a triggered release of oxaliplatin by secretory phospholipase A_2 ($sPLA_2$) [13–16]. A frequently occurring challenge, related to nanoparticulate formulation development activities, is the limited availability of liposomal test formulations (small batch sizes). This leaves only sparse amounts of material for the characterization of the formulations, in order to preserve material for investigating their performance. Accordingly, capillary-based techniques should potentially be of interest for characterizing liposomal drug delivery systems in formulation development, as CE has low sample consumption and provides rapid analysis. Albeit, the characterization and analysis of liposomes by capillary electrophoretic methods have attracted recent attention [17–21], only a few studies have utilized CE to characterize liposomal drug delivery systems. These studies focused on determining drug encapsulation [22–24], stability of the drug formulation [23,25] or binding affinity between the drug substance and the delivery system [26–28]. Different approaches

* Corresponding author. Fax: +45 35336030.
E-mail address: joe@farma.ku.dk (J. Østergaard).
[1] Present address: DermaVeris ApS, Soetoftevej 30, DK-4100 Ringsted, Denmark.

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.12.037

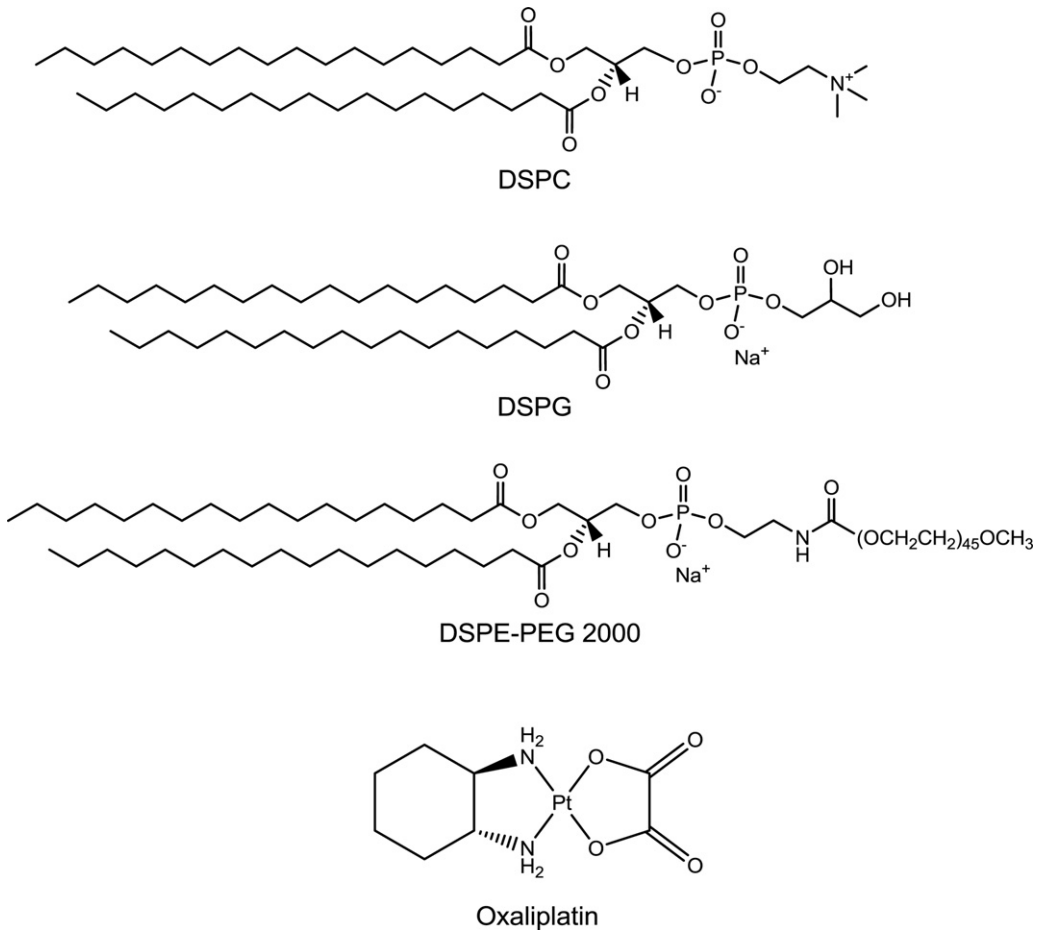

Fig. 1. Structure of oxaliplatin and the phospholipids constituting the liposomes. DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPG, 1,2-dioctadecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) monosodium salt, DSPE-PEG2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] monosodium salt.

have been taken in order to separate the free drug from the encapsulated, e.g., ultracentrifugation, size exclusion chromatography, filtration or dialysis [19]. This separation is typically followed by a disruption of the lipid bilayer, typically induced by the use of surfactants or organic solvents, in order to determine the total drug concentration in the formulation [29]. In this study, the separation step was performed by CE. Free (non-encapsulated) oxaliplatin was removed upon preparation of the liposomes by dialysis [30]. However, a small amount of free drug substance will often remain present in the drug formulation. Depending on the stability of the liposomal formulation, the propensity of drug leakage can be followed by measuring the amount of free and encapsulated drug over time. Consequently, analytical methods to determine the free and encapsulated drug are needed.

The aim of the study was to develop a rapid CE method for the characterization of an anionic model PEGylated liposomal formulation of the anti-cancer agent oxaliplatin for use in formulation development. A CE method for the determination of the entrapment and leakage of oxaliplatin from the drug formulation was developed. To assess whether CE could be a useful tool for studying drug leakage from a PEGylated liposomal formulation, accelerated drug leakage studies (oxaliplatin leakage) were performed. This was done by stressing the liposomal drug formulation by ultrasound followed by CE analysis. Results obtained by the CE method, for the determination of total oxaliplatin content in the liposomal drug formulation, were compared to measurements conducted using inductively coupled plasma mass spectrometry (ICP-MS).

2. Materials and methods

2.1. Chemicals

Calcium gluconate, paracetamol, sucrose, and Triton X-100 were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Oxaliplatin was obtained from Yingxuan Chempharm (Shanghai, China). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioctadecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) monosodium salt (DSPG), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] monosodium salt (DSPE-PEG2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Nitric acid 65% (w/w), p.a. was obtained from Merck (Darmstadt, Germany). All other chemicals and reagents were of at least analytical grade. All chemicals were used as received. Purified water prepared from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used throughout.

2.2. Preparation and characterization of liposomes

Two types of liposomal preparations were produced. Empty liposomes, without oxaliplatin, and active liposomes in which oxaliplatin was encapsulated into the liposomal structures. The liposomes were composed of DSPC, DSPG and DSPE-PEG2000 (DSPC/DSPG/DSPE-PEG2000 70/25/5, mol%). The preparation of liposomes was performed as described previously [30]. Briefly, the liposomes were prepared by the lipid film method [31] followed by hydration with a 10% (w/v) sucrose solution containing

1 mM calcium gluconate. The hydration solution for the preparation of active liposomes additionally contained 17 mg/mL oxaliplatin. Following hydration, the liposomes were extruded through a polycarbonate filter with a pore size of 100 nm (Whatman International, Maidstone, UK). Upon extrusion, free oxaliplatin was removed by dialysis against the hydration solution (without oxaliplatin). A basic characterization of liposomal preparations encompassed determination of lipid content by phosphorous analysis [32], main transition temperature by differential scanning calorimetry using a MicroCal MC-2 calorimeter (Northampton, MA, USA), and zeta potential by laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Calculation of size and size distribution based on light scattering intensity, assuming spherical particles, was performed using a Zetasizer Nano ZS.

2.3. Determination of oxaliplatin solubility

Excess oxaliplatin was added to at solution consisting of 10% (w/v) sucrose and 1 mM calcium gluconate (1 mL) and incubated in a Problot L12 hybridization oven (Labnet International Inc., Woodbridge, NJ, USA) at 25 ± 0.5 °C and at 65 ± 0.5 °C. Samples were withdrawn and filtered upon 24 and 48 h of incubation, to ensure equilibrium, using pre-heated 0.22 μm Millex-HV PVDF filters (Millipore, Bedford, MA, USA) before diluting samples with the 10% (w/v) sucrose and 1 mM calcium gluconate solution. The resulting samples were subjected to CE analysis.

2.4. Liposome analysis

2.4.1. CE instrumentation

CE experiments were conducted using a HP ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. Fused silica capillaries with an extended light path (~150 μm), and an effective length of 40 cm and an inner diameter of 50 μm were used in all experiments (Agilent Technologies). All standards and samples were introduced hydrodynamically (50 mbar for 5 s) and the applied voltage was +30 kV (~1.6 μA). UV detection was performed at 200, 214 and 254 nm and the temperature of the capillary cassette was set to 25 °C. All standards and samples were analyzed in triplicate. A solution containing 10% (w/v) sucrose and 1 mM calcium gluconate was used as the background electrolyte (BGE) for all CE experiments. Between runs, the capillary was flushed with 1% (w/v) SDS solution, 0.1 M NaOH, and background electrolyte for 2 min each [33]. Daily, the capillary was flushed with 1 M NaOH, a 1% (w/v) SDS solution, and background electrolyte for 5 min each before starting experiments. New capillaries were conditioned by flushing with 1 M NaOH, 0.1 M NaOH, and the background electrolyte for 30 min each.

2.4.2. ICP-MS instrumentation

A PerkinElmerSciex DRC-e ICP mass spectrometer (Perkin Elmer, SCIEX, Norwalk, CT, USA) equipped with a Micromist nebulizer 0.2 mL/min and a programmable temperature unit IsoMist Module LOL with standard spray chamber V2 (Glass Expansion, Melbourne, Australia) was used for platinum determination. The instrument was run and controlled by Elan Software Version 3.4 (Perkin Elmer). Pure xenon gas (Air Liquide Deutschland GmbH, Düsseldorf, Germany) was used as a collision gas. The nebulizer gas flow rate, plasma RF power, lens voltage as well as all dynamic reaction cell (DRC) parameters were optimized on a Pt standard solution. The $^{195}Pt^{+}$ isotope was monitored using DRC mode with dwell time of 250 ms, 20 sweeps, 1 reading, and 5 replicates per run.

2.4.3. Determination of free oxaliplatin concentration by capillary electrophoresis

The background electrolyte and diluted liposomal dispersions were filtered through a 0.45 μm nylon filter (SMI-Labhut Ltd, Gloucestershire, UK) before their introduction into the CE instrument. Liposomal samples, for the determination of the free oxaliplatin concentration, were diluted by a factor of 5 with BGE and analyzed without further sample preparation. A linear relationship ($r^2 > 0.995$) between normalized peak areas (area/migration time) and the concentration of oxaliplatin standards dissolved in the BGE was observed in the concentration range (0.05–0.50 mg/mL) using five different oxaliplatin concentrations. For oxaliplatin standards and liposome containing samples the RSD of normalized peak areas was generally ≤5% ($n = 3$). The limit of detection was determined to 0.014 mg/mL, calculated as 3.3 standard deviations of the response of the blank and divided by the slope of the calibration curve. The limit of quantification was 0.041 mg/mL, calculated as 10 standard deviations of the response of the blank and divided by the slope of the calibration curve.

2.4.4. Determination of total oxaliplatin concentration by capillary electrophoresis

250 μL of diluted liposomal samples (1:5 dilution in BGE), 247 μL of BGE and 3 μl of Triton X-100 (155 mM) were mixed, to provide a total concentration of Triton X-100 of 0.93 mM, before transferring samples into 0.5 mL Amicon Ultra centrifugal filters (cut-off 30 kDa, Millipore Corporation, Billerica, MA, USA) followed by centrifugation for 30 min (14,000 rcf) using a tabletop centrifuge (MiniSpin plus, Eppendorf, Hamburg, Germany). The oxaliplatin concentration in the filtrate of the samples was determined by both CE and ICP-MS (*cf.* Section 2.4.5). The calibration curve used for the total determination of oxaliplatin was prepared in the same way as the samples by mixing an oxaliplatin standard in a given concentration, Triton X-100 to a concentration of 0.93 mM, and empty liposomes with a similar lipid composition and concentration as the drug loaded liposomes followed by centrifugation and analysis of the filtrate as described above. This was done in order to avoid matrix effects. A linear relationship ($r^2 > 0.998$) between normalized peak areas (area/migration time) and the concentration of oxaliplatin was observed in the relevant concentration range (0.05–1.0 mg/mL) using five different oxaliplatin concentrations. The limit of detection was determined to 0.023 mg/mL, calculated as 3.3 standard deviations of the response of the blank and divided by the slope of the calibration curve. The limit of quantification was 0.068 mg/mL, calculated as 10 standard deviations of the response of the blank and divided by the slope of the calibration curve. The RSD was ≤8% for the standards ($n = 3$).

2.4.5. Determination of total oxaliplatin content by ICP-MS

The total oxaliplatin concentration was determined directly in liposomal samples diluted 10 times in BGE and containing 0.93 mM Triton X-100. A CEM MDS-81D microwave digestion system (CEM Corporation, NC, USA) with 3 mL CEM Teflon vials was used for sample digestion. An aliquot of 100 μL liposome sample diluted 10 times was pipetted into 3 mL CEM vials together with 700 μL of sub-boiled 65% (w/w) nitric acid. For sample digestion, a two-step microwave program was modified according to the manufacturer's instructions. After digestion and cooling the solution, deionized water was added to a final volume of 20 mL. For the quantification of platinum by ICP-MS, a calibration curve with five calibration points was made in the range of 20–500 ng/mL of oxaliplatin in 1.3% HNO_3. The squared correlation coefficient (r^2) was found to be 0.9999 for the $^{195}Pt^{+}$ isotope. The detection limit of oxaliplatin was 0.05 ng/mL and the quantitation limit was 0.19 ng/mL calculated as stated above (*cf.* Sections 2.4.3 and 2.4.4). Repeatability was ≤2.2% for the standards ($n = 8$). The oxaliplatin concentration

was furthermore determined in the filtrate after sample preparation as described above (*cf.* Section 2.4.4) to compare the two methodologies ICP-MS and CE.

2.4.6. Determination of trapping efficiency by capillary electrophoresis

The trapping efficiency (drug loading) was calculated according to:

$$\text{trapping efficiency} = \frac{C_{tot} - C_{free}}{C_{hydration}} \quad (1)$$

where C_{tot} is the total oxaliplatin concentration as determined from the concentration determined by CE taking the recovery into account. C_{free} is the free oxaliplatin concentration as determined by CE, and $C_{hydration}$ is the oxaliplatin concentration in the hydration solution (17 mg/mL). An aliquot from the liposomal preparation was withdrawn after the hydration step, but before the dialysis step, which separated the free and liposome encapsulated oxaliplatin (*cf.* Section 2.2) in order to determine the trapping efficiency. The free and total drug concentrations were determined by CE as outlined above. Liposomal samples for the determination of the free drug concentration were diluted 20 times with BGE and samples for total oxaliplatin determination were diluted 40 times with BGE before CE analysis.

2.4.7. Determination of encapsulation degree and encapsulation efficiency in final formulation

From the determination of free and total oxaliplatin concentrations in the model drug formulation the percentage of encapsulated drug was calculated according to [34]:

$$\%\,\text{encapsulated drug} = \frac{C_{tot} - C_{free}}{C_{tot}} \times 100 \quad (2)$$

The encapsulation efficiency in the final formulation was calculated as mol drug incorporated/mol lipid [35].

2.4.8. Accelerated drug leakage studies

Accelerated drug leakage studies were performed by the sonication of the oxaliplatin-containing liposomal formulation using a 2 mm probe connected to a VCX 130 ultrasonic processor (Vibra Cell, Sonics and Materials Inc., Newton, CT, USA). Samples were made by diluting the stock liposomal dispersion 10 times with a solution containing 10% (w/v) sucrose and 1 mM calcium gluconate. Separate samples of 1 mL were sonicated in 1.5 mL glass vials for 0, 15, 30, 60, 120, 240, 360 and 600 s, respectively, with on/off-cycles of sonication of 5 s/2 s at an effect of 71.5 W. The drug leakage was followed by determining the free oxaliplatin concentration in sonicated samples by CE. Furthermore, the total oxaliplatin concentration in the unsonicated drug formulation was determined by CE. Size measurements of the sonicated liposomal samples were performed by DLS.

3. Results and discussion

3.1. Liposome characteristics

PEGylated liposomes consisting of DSPC, DSPG and DSPE-PEG2000 lipids have been proposed as delivery systems for anti-cancer agents [30]. Liposomal dispersions containing oxaliplatin and 70/25/5 (mol%) DSPC/DSPG/DSPE-PEG2000 in a 10% (w/v) sucrose solution were selected as a model formulation for the present study among several investigated formulations. The total lipid concentrations in the liposomal drug formulations were 62.5 mg/mL and 61.7 mg/mL in empty and drug loaded liposomes, respectively, assuming similar incorporation efficiencies of the three phospholipids, DSPC, DSPG and DSPE-PEG2000, on a molar basis as determined by phosphorus analysis. The liposomal size distributions were monomodal with average sizes (*Z*-average) of 123.9 ± 0.5 nm and 137.9 ± 0.7 nm for empty and drug loaded liposomes, respectively, and polydispersities of 0.15 and 0.12, respectively. The liposomes were net negatively charged as the zeta potentials determined in a 10% (w/v) sucrose solution containing 1 mM calcium gluconate were −3.4 ± 0.7 mV and −5.1 ± 0.4 mV for empty and drug loaded liposomes, respectively. The main transition temperatures, as determined by DSC, were 61.5 °C and 61.8 °C for empty and drug loaded liposomes, respectively.

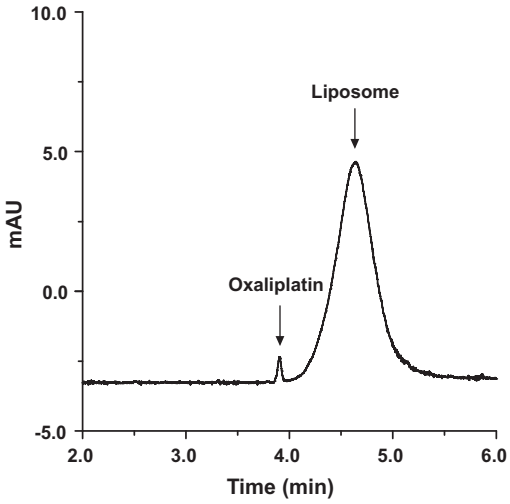

Fig. 2. Electropherogram of a PEGylated liposomal drug formulation containing oxaliplatin diluted 5 times (12.5 mg lipid/mL) for the determination of the free oxaliplatin concentration. CE conditions: BGE, 10% (w/v) sucrose containing 1 mM calcium gluconate; uncoated fused silica capillary (48.5 cm × 50 μm id, 40 cm effective length); voltage, +30 kV; capillary cassette temperature, 25 °C; hydrodynamic injection (50 mbar) for 5 s; detection at 254 nm.

3.2. Determination of free oxaliplatin by capillary electrophoresis

A key objective was to develop a fast CE method facilitating liposome characterization during development by the separation of free (non-encapsulated) oxaliplatin from the liposome encapsulated drug substance. A representative electropherogram, for the determination of the free drug concentration upon 5 fold dilution of the liposomal sample in BGE, is depicted in Fig. 2. The free, uncharged oxaliplatin was fully separated from the negatively charged liposomes (negative zeta potentials), as seen in Fig. 2. The signal of the liposomes at 254 nm is almost exclusively due to oxaliplatin as it has a much higher response factor at this wavelength compared to the empty liposomes. The liposomal dispersions were prepared in 10% (w/v) sucrose containing 1 mM calcium gluconate. Somewhat surprisingly, this uncommon background electrolyte worked well, providing constantly stable peak appearance times (RSD < 0.5%, $n = 3$) and, apparently, little capillary wall absorption of the PEGylated liposome. An additional advantage of the BGE is the minimal sample pretreatment required, as the PEGylated liposomes were analyzed in a solution which might be useful for freeze-drying and subsequent reconstitution. With a run time of 6 min (Fig. 2), the CE method was considered useful for the current purpose. Control experiments involving liposomes without oxaliplatin indicated that potential neutral impurities or degradation products originating from the lipids did not absorb at 254 nm. The free oxaliplatin concentration in the drug formulation containing 62.5 mg lipid/mL was determined to 0.082 ± 0.004 mg/mL ($n = 3$).

An obvious drawback of the CE method is the lack of separation of the uncharged oxaliplatin and any neutral degradation products.

Table 1
Total oxaliplatin concentrations in model liposomal drug formulation as determined by CE and ICP-MS ($n = 3$).

Method (sample)	Oxaliplatin concentration (mg/mL ± SD)
CE (filtrate)	1.58 ± 0.22
ICP-MS (filtrate)	1.57 ± 0.02
ICP-MS (total)	1.82 ± 0.11

Thus, the method is limited to formulation development work, and is not likely to be suitable for samples with biological content.

3.3. Determination of total oxaliplatin concentration, encapsulation degree and encapsulation efficiency

An essential parameter pertaining to the quality and performance of nanoparticulate drug delivery systems is the degree of drug encapsulated in the formulation. The total oxaliplatin content in the drug formulation was determined by inducing leakage of the encapsulated oxaliplatin by the addition of the non-ionic detergent Triton X-100. The non-ionic detergent Triton X-100 was selected as it has previously been found useful for inducing drug release from liposomes [22,23]. Preliminary studies showed that the induced drug release from the drug formulation by adding Triton X-100 in the concentration range between 0.93 mM and 12.4 mM was similar (data not shown). The recovery in the filtration step used in the developed CE method was determined from a determination of the total oxaliplatin concentration by ICP-MS. The results from the total oxaliplatin determinations are outlined in Table 1. A good agreement between average measured values of oxaliplatin in the filtrate after centrifugation for the two analytical methodologies was observed, although the variation was larger using CE as compared to ICP-MS. The recovery of oxaliplatin from the determinations using Triton X-100 for inducing drug leakage was calculated to 86.5% from the ICP-MS measurements (Table 1). The percentage of encapsulated drug in the model drug formulation was calculated to 96% from Eq. (2) using the determined free (0.082 mg/mL) and total (1.82 mg/mL) concentrations of oxaliplatin. The encapsulation efficiency was calculated to 0.066 ± 0.009 mol oxaliplatin/mol lipid. Thus, the percentage of encapsulated drug attained is comparable to what has been reported other liposomal formulations. The doxorubicin hydrochloride concentration in Doxil has been reported to 2.0 mg/mL and the encapsulation efficiency to be above 90% [36]. The encapsulation efficiency of cisplatin in PEGylated liposomes in the now abandoned SPI-77 from SEQUUS Pharmaceuticals was reported to be above 95% (1.0 mg cisplatin/mL) [37].

3.4. Determination of trapping efficiency

The trapping efficiency was determined by comparing the initial oxaliplatin concentration in the hydration media (17 mg/mL) during preparation of the liposomal formulation and the encapsulated concentration in the final drug formulation, which was 1.74 mg/mL (1.82–0.082 mg/mL). Consequently, the trapping efficiency was calculated to 10%. A sample was furthermore withdrawn from the hydrated liposomal dispersion after extrusion, in which the total oxaliplatin concentration was determined to 8.77 ± 0.42 mg/mL ($n = 4$) by CE assuming a recovery of 86.5%. Solubility studies of oxaliplatin were performed in order to evaluate whether the relatively large difference between the total oxaliplatin concentrations in the hydration solution (17 mg/mL) and in the hydrated liposomal dispersion was caused by differences in oxaliplatin solubility, at the hydration temperature (65 °C) and at room temperature. Solubility data are shown in Table 2 indicating that the differences in oxaliplatin concentration, before and after hydration, might be explained by differences in solubility at different temperatures. Additionally, the solubility studies indicate that all the oxaliplatin determinations have been performed well within the solubility range of oxaliplatin. The solubility of oxaliplatin in water at 37 °C has been reported to 7.9 mg/mL [38] strongly indicating that the solubility determinations are consistent with previous studies. In this context, fast and simple CE methods may be useful for pinpointing critical steps in the formulation design process.

Table 2
Apparent oxaliplatin solubility in a 10% (w/v) sucrose solution containing 1 mM calcium gluconate at different temperatures ($n = 3$).

Temperature	Solubility ± SD (mg/mL)
25 °C	8.0 ± 0.6
65 °C	16 ± 1.3

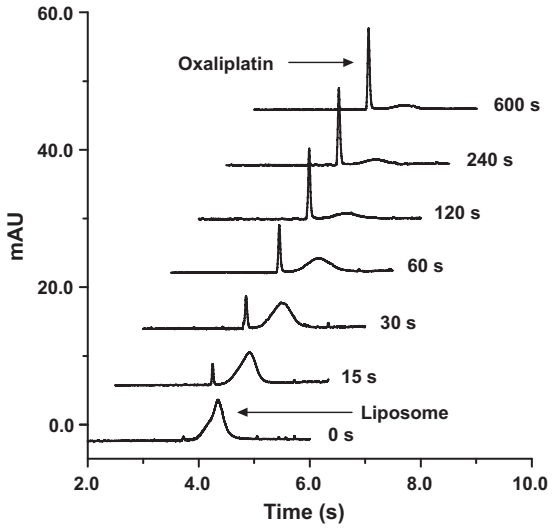

Fig. 3. Electropherograms of sonicated samples of PEGylated liposomal drug formulation (see text for details). Sonication times were between 0 and 600 s. CE conditions: BGE, 10% (w/v) sucrose containing 1 mM calcium gluconate; uncoated fused silica capillary (48.5 cm × 50 μm id, 40 cm effective length); voltage, +30 kV; capillary cassette temperature, 25 °C; hydrodynamic injection (50 mbar) for 5 s; detection at 254 nm.

3.5. Accelerated drug leakage studies investigated by capillary electrophoresis

Another important aspect of liposomal drug formulations is their propensity to leak the encapsulated cargo (drug) during storage. Upon storage for 3 months at 5 °C it was not possible to detect an increase in the free oxaliplatin concentration in the liposomal formulation by the CE method. In order to evaluate whether CE could be a feasible method for studying oxaliplatin leakage from the liposomes, sonication of the model formulation was undertaken to accelerate drug leakage. Electropherograms of liposomal samples sonicated between 0 s and 600 s are shown in Fig. 3. The decrease in the peak area of the liposome peak, and the increase in the area of the free oxaliplatin peak indicate a release of oxaliplatin from the PEGylated liposomes. The disappearance of the liposome peak does not inevitably indicate the decomposition of the liposomes, possibly, rather a release of the encapsulated oxaliplatin. The effect of sonication on oxaliplatin leakage from the liposomes is shown in Fig. 4. As seen from Fig. 4A, a complete oxaliplatin release is obtained after 4 min of sonication (5 s/2 s on/off-cycles). Parallel DLS measurements showed that the average liposome size decreases and the polydispersity increases with an increasing sonication time (Fig. 4B). The size distribution was monomodal in all the

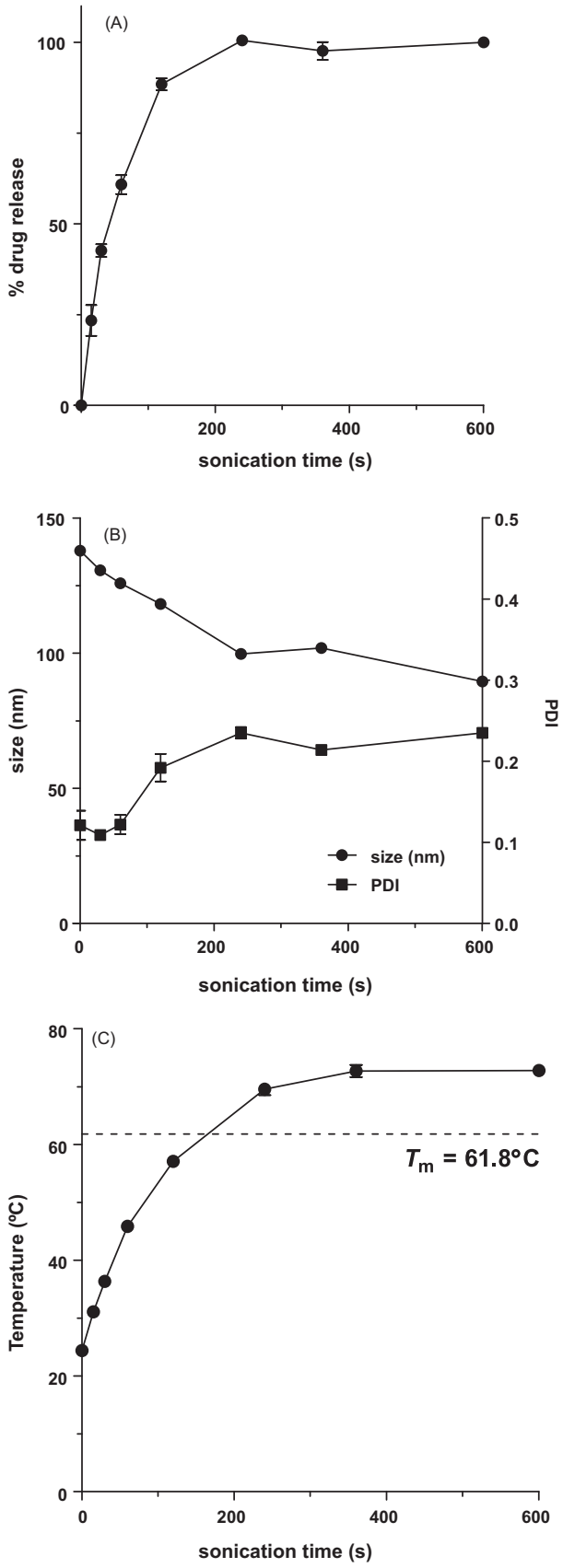

Fig. 4. (A) Oxaliplatin leakage from the PEGylated liposomal drug formulation as a function of sonication time. (B) Effect of sonication on the average size (*Z*-average) and polydispersity of liposomal formulation. (C) Sample temperature at various sonication times. Dotted line indicates the main transition temperature (T_m) of the liposomal formulation. Error bars correspond to ±SD of triplicate experiments.

sizing experiments indicating that intact vesicles are present even after 10 min of sonication. In order to evaluate whether mechanical and/or thermal effects were responsible for the drug leakage during sonication, the temperature was measured in the dilution media consisting of 10% (w/v) sucrose and 1 mM calcium gluconate at different sonication times (Fig. 4C). Sonication caused the temperature to rise to 70 °C after 4 min of sonication, which is above the main transition temperature (61.8 °C) indicating that the oxaliplatin release may also be caused by thermal effects most likely involving a phase transition of the liposomes. The average free oxaliplatin concentration taken as an average of all the measurements of the three last time points (4, 6 and 10 min) was 1.90 ± 0.04 mg/mL indicating that the oxaliplatin release was complete already after 4 min of sonication. The results may suggest that sonication could be used as a simple sample preparation procedure in order to determine the total amount of encapsulated drug in the PEGylated liposomes. This, obviously, requires that the propensity of the encapsulated drug compound (or any products generated by the sonication procedure) to bind to the intact liposomes, is low. Using affinity CE in the form of capillary electrophoresis frontal analysis and electrokinetic chromatography it was not possible to detect any interaction between oxaliplatin and the PEGylated liposomal membrane (to be reported elsewhere).

4. Conclusion

In this work CE was used to characterize a PEGylated liposomal formulation of the anti-cancer agent oxaliplatin. A simple CE method to determine free and total oxaliplatin concentrations was developed, in order to calculate drug encapsulation, encapsulation efficiency and trapping efficiency. The accuracy of determination of the total drug concentration in the model formulation was confirmed by ICP-MS. Accelerated drug leakage could also be monitored by CE. In conclusion, CE was successfully applied for the characterization of the PEGylated liposomal drug formulation and may be considered a useful tool in a drug formulation development. In addition to the well-known characteristics, low sample and background electrolyte consumption, simple sample preparation, a high degree of automation, and relatively short analysis times, the ability to analyze intact liposomes in the intended formulation media with minimal sample preparation was demonstrated.

Acknowledgements

Anders F. Vikbjerg, Sune Petersen and Mats Leeman from LiPlasome Pharma A/S are gratefully acknowledged for technical support and fruitful discussions. We thank Gunnel Karlsson at Biomicroscopy Unit, Polymer and Materials Chemistry, Chemical Centre, Lund University, Lund, Sweden for the cryoTEM work. This research was supported by the Drug Research Academy, Faculty of Pharmaceutical Sciences, University of Copenhagen, Denmark.

References

[1] M.J. McKeage, New-generation platinum drugs in the treatment of cisplatin-resistant cancers, Expert Opin. Investig. Drugs 14 (2005) 1033–1046.
[2] A.M. Di Francesco, A. Ruggiero, R. Riccardi, Cellular and molecular aspects of drugs of the future: oxaliplatin, Cell. Mol. Life Sci. 59 (2002) 1914–1927.
[3] M.E. Davis, Z.G. Chen, D.M. Shin, Nanoparticle therapeutics: an emerging treatment modality for cancer, Nat. Rev. Drug Discov. 7 (2008) 771–782.
[4] D. Sutton, N. Nasongkla, E. Blanco, J. Gao, Functionalized micellar systems for cancer targeted drug delivery, Pharm. Res. 24 (2007) 1029–1046.
[5] W.C. Zamboni, Liposomal, nanoparticle, and conjugated formulations of anticancer agents, Clin. Cancer Res. 11 (2005) 8230–8234.
[6] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, Science 303 (2004) 1818–1822.
[7] D.D. Lasic, Liposomes: From Physics to Applications, Elsevier, Amsterdam, 1993.
[8] J.M. Metselaar, E. Mastrobattista, G. Storm, Liposomes for intravenous drug targeting: design and applications, Mini. Rev. Med. Chem. 2 (2002) 319–329.

[9] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2005) 145–160.
[10] T.L. Andresen, S.S. Jensen, K. Jorgensen, Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release, Prog. Lipid Res. 44 (2005) 68–97.
[11] T.M. Allen, Long-circulating (sterically stabilized) liposomes for targeted drug delivery, Trends Pharmacol. Sci. 15 (1994) 215–220.
[12] D.M. Vail, M.A. Amantea, G.T. Colbern, F.J. Martin, R.A. Hilger, P.K. Working, Pegylated liposomal doxorubicin: proof of principle using preclinical animal models and pharmacokinetic studies, Semin. Oncol. 31 (2004) 16–35.
[13] T. Abe, K. Sakamoto, H. Kamohara, Y. Hirano, N. Kuwahara, M. Ogawa, Group II phospholipase A2 is increased in peritoneal and pleural effusions in patients with various types of cancer, Int. J. Cancer 74 (1997) 245–250.
[14] T.L. Andresen, S.S. Jensen, T. Kaasgaard, K. Jorgensen, Triggered activation and release of liposomal prodrugs and drugs in cancer tissue by secretory phospholipase A2, Curr. Drug Deliv. 2 (2005) 353–362.
[15] J. Davidsen, K. Jorgensen, T.L. Andresen, O.G. Mouritsen, Secreted phospholipase A(2) as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue, Biochim. Biophys. Acta 1609 (2003) 95–101.
[16] B.P. Kennedy, C. Soravia, J. Moffat, L. Xia, T. Hiruki, S. Collins, S. Gallinger, B. Bapat, Overexpression of the nonpancreatic secretory group II PLA2 messenger RNA and protein in colorectal adenomas from familial adenomatous polyposis patients, Cancer Res. 58 (1998) 500–503.
[17] G. Bilek, L. Kremser, D. Blaas, E. Kenndler, Analysis of liposomes by capillary electrophoresis and their use as carrier in electrokinetic chromatography, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 841 (2006) 38–51.
[18] A. Gomez-Hens, J.M. Fernandez-Romero, The role of liposomes in analytical processes, Trend Anal. Chem. 24 (2005) 9–19.
[19] A. Gomez-Hens, J.M. Fernandez-Romero, Analytical methods for the control of liposomal delivery systems, Trend Anal. Chem. 25 (2006) 167–178.
[20] S.K. Wiedmer, R. Shimmo, Liposomes in capillary electromigration techniques, Electrophoresis 30 (2009) S240–S257.
[21] R.L. Owen, J.K. Strasters, E.D. Breyer, Lipid vesicles in capillary electrophoretic techniques: characterization of structural properties and associated membrane–molecule interactions, Electrophoresis 26 (2005) 735–751.
[22] D. Chen, D.L. Cole, G.S. Srivatsa, Determination of free and encapsulated oligonucleotides in liposome formulated drug product, J. Pharm. Biomed. Anal. 22 (2000) 791–801.
[23] N. Griese, G. Blaschke, J. Boos, G. Hempel, Determination of free and liposome-associated daunorubicin and daunorubicinol in plasma by capillary electrophoresis, J. Chromatogr. A 979 (2002) 379–388.
[24] P. Perjesi, T. Kim, A.D. Zharikova, X. Li, T. Ramesh, J. Ramasubbu, L. Prokai, Determination of clodronate content in liposomal formulation by capillary zone electrophoresis, J. Pharm. Biomed. Anal. 31 (2003) 929–935.
[25] M.J. Orchard, I.P. Collin, M. Bushell, The use of capillary electrophoresis with entangled polymer matrix to analyse plasmid DNA and a cationic lipid/cholesterol liposome: DNA complex, Chromatographia 50 (1999) 181–187.
[26] J. McKeon, M.G. Khaledi, Evaluation of liposomal delivery of antisense oligonucleotide by capillary electrophoresis with laser-induced fluorescence detection, J. Chromatogr. A 1004 (2003) 39–46.
[27] E.H. Moeller, B. Holst, L.H. Nielsen, P.S. Pedersen, J. Østergaard, Stability, liposome interaction, and in vivo pharmacology of ghrelin in liposomal suspensions, Int. J. Pharm. 390 (2010) 13–18.
[28] J. Østergaard, E.H. Moeller, Ghrelin–liposome interactions: characterization of liposomal formulations of an acylated 28-amino acid peptide using CE, Electrophoresis 31 (2010) 339–345.
[29] K.A. Edwards, A.J. Baeumner, Analysis of liposomes, Talanta 68 (2006) 1432–1441.
[30] A.F. Vikbjerg, S.A. Petersen, F. Melander, J.R. Henriksen, K. Jørgensen, Liposomes for drug delivery and methods for preparation thereof, WO 2009/141450 A2 (2009).
[31] V.P. Torchilin, V. Weissig, Liposomes. A Practical Approach, Oxford University Press, New York, 2003.
[32] P.S. Chen, T.Y. Toribara, H. Warner, Microdetermination of phosphorus, Anal. Chem. 28 (1956) 1756–1758.
[33] U. Franzen, L. Jorgensen, C. Larsen, N.H. Heegaard, J. Østergaard, Determination of liposome-buffer distribution coefficients of charged drugs by capillary electrophoresis frontal analysis, Electrophoresis 30 (2009) 2711–2719.
[34] S. Vemuri, C.T. Rhodes, Separation of liposomes by a gel filtration chromatographic technique: a preliminary evaluation, Pharm. Acta Helv. 69 (1994) 107–113.
[35] P. Kallinteri, D. Fatouros, P. Klepetsanis, S.G. Antimisiaris, Arsenic trioxide liposomes: encapsulation efficiency and in vitro stability, J. Liposome Res. 14 (2004) 27–38.
[36] J. Vaage, D. Donovan, P. Uster, P. Working, Tumour uptake of doxorubicin in polyethylene glycol-coated liposomes and therapeutic effect against a xenografted human pancreatic carcinoma, Br. J. Cancer 75 (1997) 482–486.
[37] J. Vaage, D. Donovan, E. Wipff, R. Abra, G. Colbern, P. Uster, P. Working, Therapy of a xenografted human colonic carcinoma using cisplatin or doxorubicin encapsulated in long-circulating pegylated stealth liposomes, Int. J. Cancer 80 (1999) 134–137.
[38] Y. Kidani, K. Inagaki, M. Iigo, A. Hoshi, K. Kuretani, Antitumor activity of 1,2-diaminocyclohexane–platinum complexes against sarcoma-180 ascites form, J. Med. Chem. 21 (1978) 1315–1318.