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ARTICLE

New oral liposomal vitamin C formulation: properties and bioavailability

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ABSTRACT

Vitamin C is the exogenous compound necessary for a variety of metabolic processes; therefore, the efficient delivery is critical for the maintenance of body homeostasis. Vitamin C pharmacokinetics and low quantities in processed foodstuff, necessitates its continuous supplementation. In the paper, we present the new liposomal formulation of vitamin C free of harmful organic solvents. The formulation was quantitatively characterized with respect to its chemically composition and nano-structuring. The vitamin C accessibility to cells from the formulation was evaluated using evidence derived from experiments performed on cell cultures. Finally, the enhanced bioavailability of vitamin C from the formulation was demonstrated in the medical experiment.

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Liposome; vitamin C; efficacy; bioavailability; stability

1. Introduction

Vitamin C is a compound essential for maintaining cellular homeostasis in mammals. However, the endogenous adjustments of vitamin C in humans are not possible because of a mutation that eliminated its endogenous availability. Consequently, it has to be acquired from the diet. The lack of direct feedback between the body requirement and the intrinsic supply of ascorbic acid causes a large proportion of the human population to be in a state of various levels of permanent or transient deficiencies (Padayatty *et al.* 2004, Lindblad *et al.* 2013, Padayatty and Levine 2016). As vitamin C is directly involved in many vital processes, such as protection against excess of reactive oxygen species, maintenance of iron homeostasis and gene expression control, therefore even moderate deficiency can have serious health consequences (Salganik 2001, Fraga and Oteiza 2002, Babaev 2010, Finck *et al.* 2014, Lane and Richardson 2014, Young *et al.* 2015). This can be easily prevented by the diet adjustment and/or additional supplementation. The supplementation is safe since kidneys remove any excess of vitamin C preventing overdose. The recommended daily intake of vitamin C for a healthy person has been determined to be 500 mg/day (Savini *et al.* 2005, Harrison *et al.* 2014, Paschalis *et al.* 2016). The recommendation does not account for the elevated demand during physical or psychological stress (May and Qu 2005, Nair *et al.* 2016). For example, it has been demonstrated that it is beneficial to increase the dose of vitamin C in the course of oncological therapies (Du *et al.* 2012, Fritz *et al.* 2014), during the treatment of skin diseases (May and Qu 2005, Stamford 2012, Kishimoto *et al.* 2013), in the reduction of stroke effects (Spector 2016) or in the elimination of certain disorders of the digestive system (Aditi and Graham 2012).

The delivery of vitamin C, using the oral delivery route, requires reduction of the rate of its degradation in the gut and facilitation of its absorption. Vitamin C is usually administered orally in the crystalline form or as a solution, which makes it susceptible to degradation in the gastrointestinal tract, especially in the presence of metal ions (Michels and Frei 2013). Degradation of vitamin C can be effectively reduced by its association with the hydrophilic–hydrophobic interface, which can be provided by lipid aggregates (Nagle and Tristram-Nagle 2000, Pastoriza-Gallego *et al.* 2012, Wechtersbach *et al.* 2012).

Lipid aggregates, such as liposomes, are well suited for this purpose. They reduce the vitamin C degradation in the gastrointestinal tract, slow down its release and enhance absorption (Hickey *et al.* 2008, Wechtersbach *et al.* 2012). Liposomes also mitigate possible perturbances of gastrointestinal tract functioning, what enables application of elevated doses of vitamin C for extended periods of time. In addition, it is not to be overlooked that lipids, phosphatidylcholines in particular, are an important component of a balanced diet with documented positive effects on the patient overall wellbeing (Alvarez and Rodriguez 2000, Keller 2001, Kullenberg *et al.* 2012, Blesso 2015, Garcia and Aguero 2015, van der Veen *et al.* 2017). All that have stimulated numerous works leading to the development of liposomal formulations of vitamin C for varieties of applications (Hickey *et al.* 2008,

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Xie and Ji 2008, Marsanasco *et al.* 2011). The manufacturing of liposomal preparations on an industrial scale requires strict process control of both chemical and physicochemical parameters, which make the production very challenging (van Nieuwenhuyzen and Szuhaj 1998, van Nieuwenhuyzen and Tomas 2008). It is essential, not only to ensure the proper and stable chemical composition of the preparation but also its nano-scale structuring. This means, that the analysis of liposomal preparations requires the application of advanced measurement methods such as: high-performance liquid chromatography (HPLC), dynamic light scattering (DLS), and transmission electron cryomicroscopy. The additional challenge is the elimination of organic solvents from the formulation and preferably from the production process as well. In the paper, we present new liposomal formulation of uniform lipid vesicle population without application of any undesirable organic solvent, with long-term stability. The developed new formulation significantly enhances vitamin C bioavailability and maintains its efficacy on the cellular level.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (Phospholipon 90G) was purchased from Lipoid GmbH (Ludwigshafen, Germany) and rapeseed lecithin from Somar (Wąchock, Poland). Sodium ascorbate was of pharmaceutical grade and was obtained from Brenntag (Kędzierzyn-Koźle, Poland) and glycerine was obtained from TechlandLab (Tarnobrzeg, Poland). Water in all solutions was highly purified with a conductivity of 0.056 uS/cm – app. 17.86 MΩ (AquaEngineering, Warszawa, Poland). Ammonium formate and organic solvents such as n-hexane, 2-propanol, and acetonitrile were of HPLC grade and were purchased from VWR International (Radnor, PA, USA).

2.2. Preparation of liposomal formulation

Liposomal formulation of vitamin C was prepared using the pharmacologically accepted glycerine, as a solvent for lipids. The other unique feature of the formulation is high content of lipids, i.e. more than 20% w/w. The high lipid content results in the high encapsulation efficiency. Liposomes were formulated by mixing two solvents: glycerine containing lipids (1:1 w/w) and the aqueous solution containing sodium ascorbate, with the API concentration equals 20% w/w. The uniform population of liposomes were formed spontaneously upon mixing. The high content of lipids (more than 20%) in the mixture results with structured aqueous phase, characterized by high viscosity and gel-like consistency. For control studies, the aqueous solution of sodium ascorbate was exchanged for pure water. Liposomes for HPLC method validation were prepared by hydration with 20 mM ammonium formate, pH 3.2 and 33.3% (w/w) of sodium ascorbate. After each preparation of liposome suspension, the size and polydispersity was measured to confirm the creation of monodisperse liposome population.

2.3. Samples preparation for HPLC analysis

The hydrophobic and hydrophilic components of the formulation were separated by the Bligh–Dyer method (Bligh and Dyer 1959) with some critical modifications. The volume of the aqueous phase was highly increased to prevent the crystallization of ascorbic acid induced by methanol. Specifically, the sample and methanol were mixed in the volume ratio 1:1. Two volumes of chloroform were added and the sample was intensively shaken. Next, the sample was 19 times diluted in 20 mM ammonium formate buffer (pH 3.2) and vortexed again. Finally, the sample was centrifuged at 2500 rpm for 10 min to speed up the separation of phases. The upper aqueous phase with vitamin C was then taken for the measurement. With this protocol, method recovery was more than 99%, which was established in the separate experiment.

2.4. HPLC analysis

HPLC analyses were performed with Knauer system (Knauer GmbH, Berlin, Germany) consisting of Knauer Azura Pumps, P2.1 ceramic head, Optimas Autosampler with 96 positions and Knauer Azura CT2.1 thermostat. All reagents were filtered through 220 nm pore size filter and degassed prior the measurement.

2.4.1. Determination of vitamin C concentration in liposomes

Vitamin C content in liposomes was determined by HPLC equipped with a UV-VIS detector Knauer Azura UVD2.1S. A Knauer LiChrospher 100-5 Diol column (125 × 4 mm^2) was used to separate the compound from other components. The column temperature was set to 20 °C. The mobile phase consisted of a 10% (v/v) solution of 20 mM ammonium formate acidified with formic acid to pH 3.2 and 90% (v/v) acetonitrile. The flow during analysis was 1 mL/min. Standard samples for calibration curves were prepared by dissolving the appropriate amount of sodium ascorbate in 20 mM ammonium formate buffer (pH 3.2).

2.4.2. Determination of lipid concentration in liposomes

Analysis of phosphatidylcholine content was performed by HPLC equipped with evaporative light scattering detector (HPLC-ELSD) (Letter 1992) and was carried out according to the method L-M-HPLC-SPC-3E/01 provided by Lipoid GmbH (Ludwigshafen, Germany) (Lipoid GmBH, 2006). The method utilizes Knauer LiChrospher 100-5 Diol column (125 × 4 mm^2) and the measurement was made using a hydrophobic phase gradient of n-hexane–isopropanol in the aqueous phase containing acetic acid and triethylamine. The method was adjusted to the ELSD Alltech 3300 detector (Buchi, Flawil, Switzerland), what improved signal quality. This modification involved the elimination of phase modifiers, namely acetic acid and triethylamine. Modifications did not affect parameters of the recorded signals, so the full width at half maximum (FWHM) was 0.1020 ± 0.0034 (relative standard deviation (RSD)=3.3%) and the measured signal height

equals to 352 ± 14 (RSD = 4.1%). The measurement was carried out in a mobile phase gradient consisting of two components. Phase A consists of n-hexane and 2-propanol mixed in the volume ratio of 830:170 and phase B consists of 2-propanol and water mixed in the volume ratio of 340:55.3. The mobile phase compositions are shown in supplementary data Table S1.

2.5. Liposome size distribution and zeta potential determination

The DLS technique allows the measurement of zeta potential and the size distribution of aggregates in the aqueous suspension. For that purpose, highly concentrated liposomal vitamin C samples were diluted 60 times with isosmotic solution prior to the analysis. Measurement was performed in 1 cm polystyrene cuvettes. Size measurements were performed using ZetaSizer Nano ZS (Malvern, UK).

2.6. Determination of the vitamin C encapsulation efficiency

The encapsulation efficiency (%EE) of vitamin C in liposomes was determined using the ultrafiltration through membranes with 50 kDa cutoff mass (SpectrumLabs, Rancho Dominguez, CA) followed by HPLC UV-VIS determination of vitamin C content in the permeate. The stability of liposomes during ultrafiltration was controlled with DLS measurements. Vitamin C encapsulation efficiency in percentiles was calculated according to the formula:

$$EE_{vitC} = (1 - m_{free}/m_{total}) \times 100$$

where m_{free} is the mass of vitamin C in the permeate and m_{total} is the total mass of vitamin C in the sample.

2.7. Cryogenic transmission electron microscopy imaging

Cryogenic transmission electron microscopy (cryo-TEM) images were obtained using a Tecnai F20 TWIN microscope (FEI Company, Hillsboro, OR, USA) equipped with a field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Eagle 4k HS camera (FEI Company, Hillsboro, OR, USA) and processed with TIA software (FEI Company, Hillsboro, OR, USA). Specimen preparation was done by verification of the aqueous solutions on grids with a holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Großlöbichau, Germany). Prior to use, the grids were activated for 15 s in oxygen plasma using a Femto plasma cleaner (Diener Electronic, Ebhausen, Germany). Cryo samples were prepared by applying a droplet (3 μL) of the solution to the grid, blotting with filter paper and rapid freezing in liquid ethane using a fully automated blotting device Vitrobot Mark IV (FEI Company, Hillsboro, OR, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a cryo-TEM-holder Gatan 626 (Gatan Inc., Pleasanton, CA, USA) and analysed in the TEM at –178 °C.

2.8. Rheology studies

The rheological characteristic of the novel liposomal formulation was measured using Brookfield rheometer DV2T of the cone-plate type, with cone type CPA-51Z. The measurement chamber was thermostated at 25 °C by circulating water bath (VWR).

2.9. Comparative studies of liposomal vs. free vitamin C cell cytotoxicity

The experiments were performed on two breast cancer cell lines SKBR3, MCF7 and the healthy BJ cell line as a control. All cell cultures were purchased from the American Type Culture Collection (Manassas, VA, USA). Survival of the cells in the presence of high concentrations of sodium ascorbate (0.4–5 mM) in both free and liposome form was determined with protocols described elsewhere (Karlsen *et al.* 2005). In short, cells were cultured in media containing 10% FBS obtained from Thermo Fisher Scientific (Waltham, MA, USA) at 37 °C under 5% CO_2. Sodium ascorbate and liposomal formulation of sodium ascorbate were diluted in culture medium prior to the addition to cells. Cell cultures were maintained using following reagents: RPMI medium, DMEM medium, penicillin, streptomycin, L-glutamine 200 mM, 0.25% trypsin–EDTA purchased from Thermo Fisher Scientific (Waltham, MA, USA) and PBS CaMg purchased from Corning (Corning, NY, USA).

The cell survival was determined with the MTT test, performed according to ISO EN ISO 10993-5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Cells were grown on 96-well plates for 48 h then the sodium ascorbate was added (the final concentration was in the range from 0.4 mM to 5 mM). After one-hour incubation, the medium was withdrawn and the culture was supplemented with a 100 μL of fresh medium. The cells were incubated for another 22 h, followed by the addition of the MTT solution in PBS without magnesium and calcium ions. Concentrations of MTT in tested cultures were 330 μg/mL. After 2.5 h incubation, MTT was removed; the cells were supplemented with 100 μL DMSO (POCH, Gliwice, Poland) and stirred to dissolve the formazan crystals. After the dissolution of crystals, the formazan absorbance was measured with SPECTROStar Nano spectrometer (BMG Labtech, Cary, NC, USA).

2.10. Bioavailability studies of liposomal and free vitamin C formulations

The aim of the study was to compare the profiles of vitamin C serum concentration in healthy volunteers after the single oral administration either in a liposomal suspension or as an aqueous solution. After obtaining written consent, 20 healthy volunteers (10 women and 10 men aged 31–65) were included in the study. Volunteers under 18 years of age, pregnant women, with the history of renal failure and gastrointestinal disorders were excluded from the study. Volunteers were preconditioned by fasting for 12 h before the test. Each

participant was equipped with a peripheral venous catheter, which allowed multiple blood withdrawals within the timeframe of the study. Prior to the main study, the blood samples were drawn to determine the initial vitamin C concentration. Each participant was administered 10 g of vitamin C, either in the free or in the liposomal form, in one single dose, dissolved/suspended in 250 mL of water. Following vitamin C administration, blood samples were taken at 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, and 360 min. Each time 4 mL of blood was transferred to the test tube, containing heparin as an anticoagulant, as recommended by Karlsen *et al.* (2005). Vitamin C concentrations in blood samples were determined within 48 h after collection using HPLC equipped with UV/VIS detector. Blood samples were stored at 4 °C before analysis. Studies were carried out by the Research and Development Centre at the Specialized Hospital in Wrocław.

2.11. Determination of vitamin C concentrations in blood samples

Determination of vitamin C concentrations in blood samples was performed according to the method described by Karlsen *et al.* (2005). Specifically, blood samples were centrifuged at 15 000 rpm for 5 min. 500 μL of serum was then taken and mixed with a 10% TCA solution in a 1:1 ratio followed by centrifugation to remove precipitated proteins. Next, the supernatant was passed through a syringe filter with the pore diameter of 220 nm. Resulting solutions were analysed for vitamin C content. The calibration curves were generated individually for each patient by adding the predetermined amounts of sodium ascorbate solution to 1 mL of the whole blood followed by the sample treatment as described above. The recovery rate was determined in the separate experiment using samples prepared by the addition of a known amount of vitamin C to the plasma using the procedure described above. The following parameters were calculated from the raw data: AUC, T_{max}, C_{max}, and $T_{1/2}$ using Qtiplot data analysis program.

2.12. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences between the groups of cells in toxicity tests were analysed by one-way ANOVA, bioavailability data were analysed by independent *t*-test. A value of $p < 0.05$ was considered significant. For statistical analysis, Qtiplot program was used.

2.13. Ethical approvals for the study

All procedures involving human subjects/patients were approved by the Bioethical Commission at the Research and Development Centre at the Specialized Hospital in Wrocław number: KB/5/2016. Written informed consent was obtained from all subjects. Liposomal vitamin C, classified as a dietary supplement, was manufactured by Lipid Systems Ltd. (Wrocław, Poland) under conditions meeting HACCP requirements in accordance with the European Parliament Regulation No. 852/2004 from April 29 2004 (Journal of Laws EU Office of 2004 as amended) and the Act on Food and Nutrition Safety from August 25 2006 (Journal of Laws from 2015, item 594).

3. Results

3.1. Stability of liposomal vitamin C

For liposomal vitamin C stability determination, the following parameters were monitored: liposome size distribution and zeta potential, vitamin C and phospholipid contents, vitamin C encapsulation efficiency and rheology. Stability of the liposomal vitamin C was determined for six series of samples and a summary of analytical data is presented in Table S2 (Supplementary data). Figure 1(A) shows the set of typical distributions of liposome sizes. The quality of the correlation function fitting to the experimental data is shown in Figure 1(B). Samples for stability testing were stored under intermediate stability storage conditions, namely at temperature $T = 30\ ^{\circ}C \pm 2\ ^{\circ}C$ and relative humidity $RH = 65 \pm 5\%$. Measurements were performed once a month. Since the DLS is an indirect method of the particle size distribution determination, the liposomal vitamin C formulation was visualized also with cryo-TEM technique. Based on the obtained images, the size distribution and morphology of liposomes were determined. Examples of cryo-TEM images obtained for the liposomal vitamin C suspension are shown in Figure 1(C). For quantitative analysis, 20 different cryo-TEM images were analysed with Image J program and the mean liposomal diameter was determined as 180 ± 30 nm. This result is in a good agreement with the value of 168 ± 25 nm as determined with DLS technique.

The physicochemical characterization of the liposomal vitamin C included also the quantitative determination of the sodium ascorbate and phosphatidylcholine content. Since the liposomal formulation cannot be considered as a simple solution, the quantitative determination of vitamin C in liposomal formulations was based on the modified Bligh–Dyer separation method (Bligh and Dyer 1959). Dilution of the hydrophilic phase with 20 mM ammonium formate at pH 3.2, following the lipid extraction step, resulted in recovery levels reaching 99.21%±0.52%. Examples of chromatograms and resulting calibration curve along with the recovery levels are presented in Figure 2. Concentrations of sodium ascorbate were determined as areas under the peak obtained by HPLC analysis.

The new liposomal formulation shows gel-like behaviour without addition any gelling substance. The dependence of liposome gel viscosity (log μ) on the share rate (log $\dot{\gamma}$) is presented in Figure 3. The viscosity decrease with share rate indicates that the gel behaves as a thinning system, which can be quantitated using the Ostwald model (Tadros 2004). The model states that $\mu = k\dot{\gamma}^{n-1}$, where *k* and *n* are consistency index and shear thinning index, respectively. Fitting experimental data to the model shows that $n = 0.8$ as expected for pseudoplastic material. The rheological

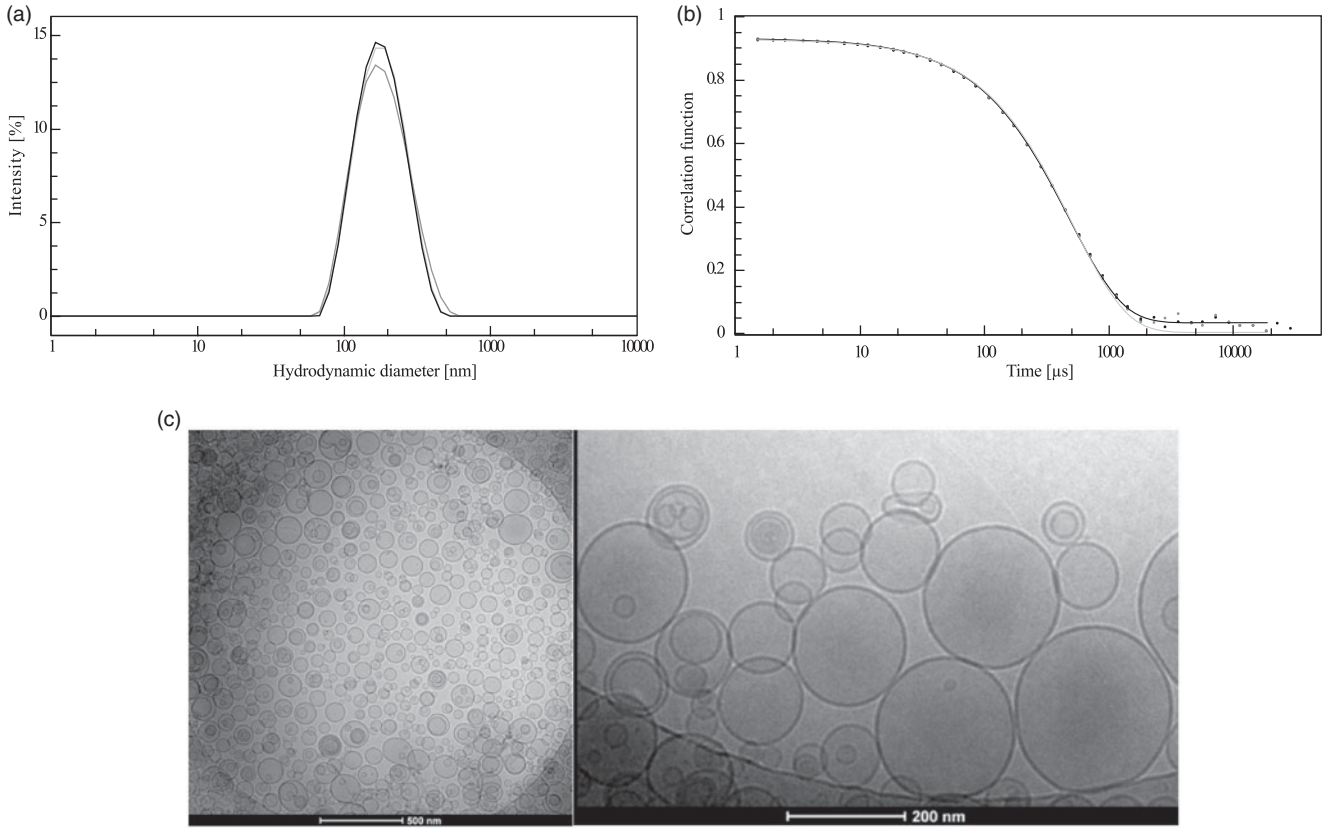

Figure 1. Examples of size distributions of liposomes loaded with sodium ascorbate as measured with the dynamic light scattering (DLS) technique and determined from images acquired with cryo-TEM microscopy. Panel a shows size distributions (expressed as hydrodynamic diameters) of liposomes with vitamin C. Panel b shows correlation curves calculated for samples presented in panel a. Experimental points were fitted with the correlation curve calculated for a single-population liposome suspension (continuous line). Panel c shows examples of cryo-TEM images using low (left image) and high (right image) resolutions.

properties of the new liposome preparation are unique and not possible to obtain using other preparation procedures.

3.2. Cytotoxicity studies

In order to determine the sodium ascorbate efficacy on the cellular level the toxicity was used as a quantitative measure. MTT tests in the cell cultures exposed to sodium ascorbate aqueous solutions and its liposomal formulation were performed. The relationship between the concentration of sodium ascorbate, incubation time and the type of cell line and cytotoxicity for sodium ascorbate aqueous solution, its liposomal formulation and liposomes without sodium ascorbate are presented in Figure 4. The experiment shows that there is no difference between the two formulations with the respect to effect on tested cells. Following one-hour incubation, both sodium ascorbate formulations were not toxic to the reference cell line (BJ) at concentrations reaching 5 mM, whereas the effect on cancerous cell lines (SKBR3 and MCF3) was already significant at concentration higher than 1 mM. This result is in good agreement with data presented elsewhere (Chen *et al.* 2005, Aguilera 2016). In addition, it has been demonstrated that the new liposome formulation alone is not toxic and that it does not interfere with the ascorbic acid activity. When the exposure time was extended to 3 h, the effect on all cells types was stronger regardless on formulation used.

3.3. Bioavailability studies

Figure 5 shows the dependence of sodium ascorbate concentration in blood on time, determined for two groups of persons, following the oral intake of 10 g of sodium ascorbate as an aqueous solution or as a liposome formulation. In the liposomal vitamin C treatment arm, vitamin C_{max} reaches higher values than in the aqueous solution of vitamin C treatment arm (303 μμ vs. 180 μμ). In addition, for liposomal formulation, the delay time of the maximum vitamin C blood concentration (T_{max}) is longer by approximately 1 h when compared to the free form (T_{max}=180 min vs. 96 min). The increased half-life ($t_{1/2}$>6 h vs. $t_{1/2}$=4 h) and elevated AUC (81 570 μμ*min vs. 45 330 μμ*min) indicate that the presence of liposomes enhances bioavailability of vitamin C.

4. Discussion

Effective vitamin C homeostasis requires two opposing fluxes (supply and elimination), which ensure the maintenance of its concentration at various body locations within physiological ranges. In humans, any excess of vitamin C is efficiently eliminated by kidneys. However, since there is no endogenous source of vitamin C, the necessary supply for the metabolic consumption and/or any temporally elevated demand can only be compensated by diet adjustments. When the excessive concentration of vitamin C is used for therapeutic purposes, the dose should exceed the excretion

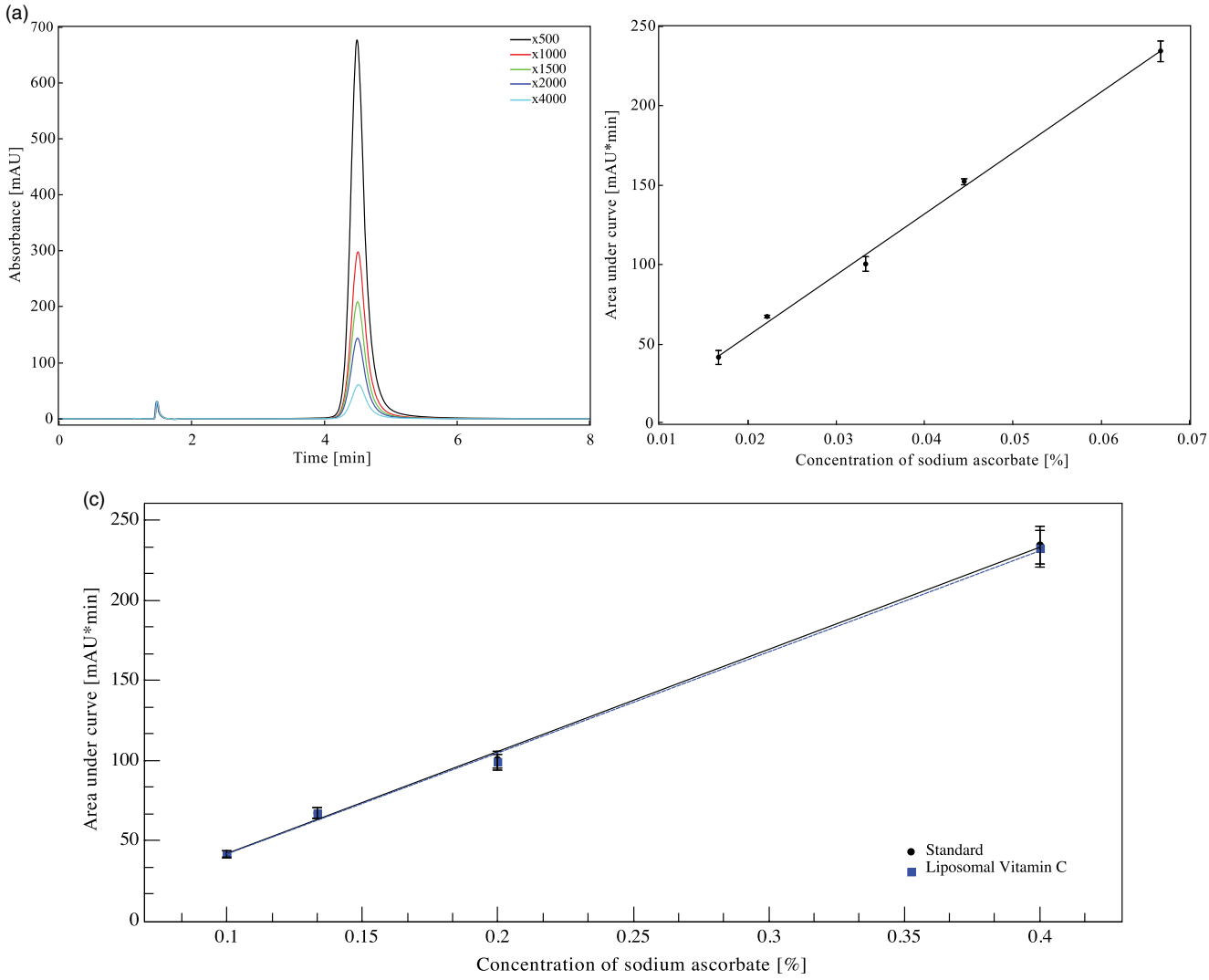

Figure 2. HPLC analysis of vitamin C concentration in serum. Panel a shows chromatograms obtained for a series of calibration samples containing sodium ascorbate encapsulated in liposomes after Blight-Dyer extraction procedure. A plot of areas under peaks, for series of sodium ascorbate concentrations constructed from chromatograms from panel a, is presented in panel b. Panel c shows the dependence of areas under the peaks derived from chromatograms obtained for two sets of experimental data used for the determination of the sodium ascorbate recovery rate; values determined for sodium ascorbate from simple solution (circles) and from liposomal formulation (squares) extracted using modified Blight-Dyer protocol. Samples after Blight-Dyer extraction contained the same amount of sodium ascorbate but were diluted 20-fold with ammonium formate at pH 3.2.

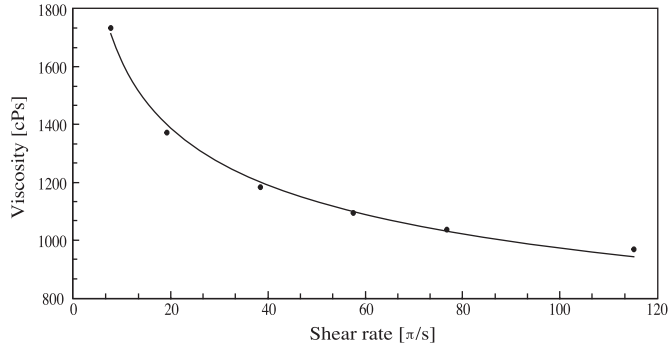

Figure 3. The dependence of the liposomal gel viscosity on the shear rate. Experimental points were fitted with the power law model (continuous line) $\mu = k\dot{\gamma}^{n-1}$. The determined value of n equals to 0.8 indicates that liposomal gel is a pseudoplastic system (Tadros 2004).

capacity of the renal system. This is the reason why the therapeutic vitamin C is delivered intravenously, so the high serum concentration can be reached and maintained for an extended period of time (Padayatty *et al.* 2004, Wilson *et al.* 2014). The inability to produce endogenous vitamin C, changing demand and uncertain intake from the diet may produce deficiencies, which can be easily eliminated by the effective supplementation (Padayatty and Levine 2016). The vitamin C blood concentration, in addition to supply and elimination, also depends on redistribution. The majority of vitamin C in the human body is inside cells (over 97%), whereas only a small fraction of it is found in extracellular fluids. High concentrations of vitamin C inside cells are possible thanks to specific sodium-dependent vitamin C transporters (SVCT1 and SVCT2). The intricate system of ascorbic acid fluxes in the human body prevents excessive fluctuation of its concentrations inside cells as required by metabolic processes. In order to affect the homeostatic balance, using a convenient oral delivery route, the vitamin C intake should be sufficiently high and preferable extended in time. To this end, the high concentration of vitamin C in the digestive tract should be maintained for extended periods of time so it would be available for absorption and would not require

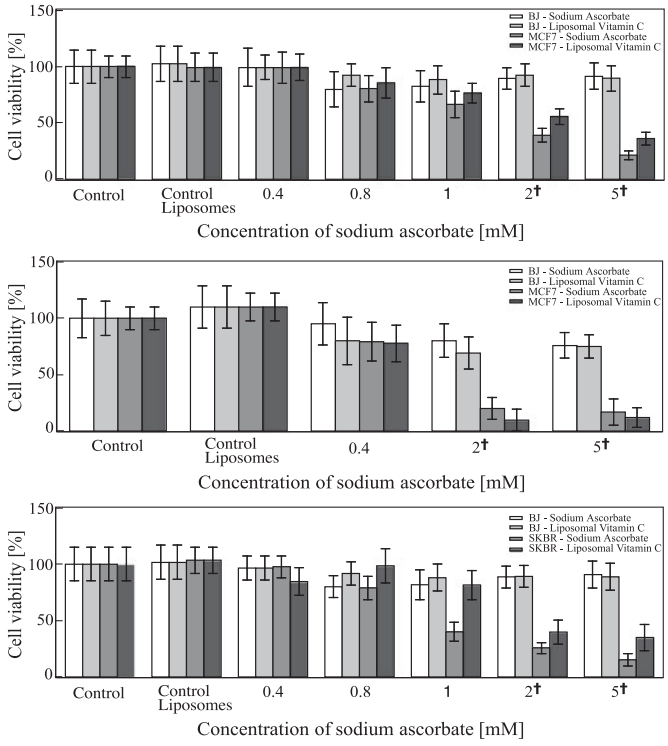

Figure 4. Effect of the sodium ascorbate concentration in the aqueous solution (white and grey) and in liposomes (light grey and black) on the survival rate of MCF7 cancer cell line and healthy BJ cells. Panel a shows data obtained after one-hour incubation whereas panel b after three-hours incubation. Panel c shows the survival rate of SKBR3 cancer cell line and healthy BJ cells after one-hour incubation. †Values are significantly different ($p < 0.05$).

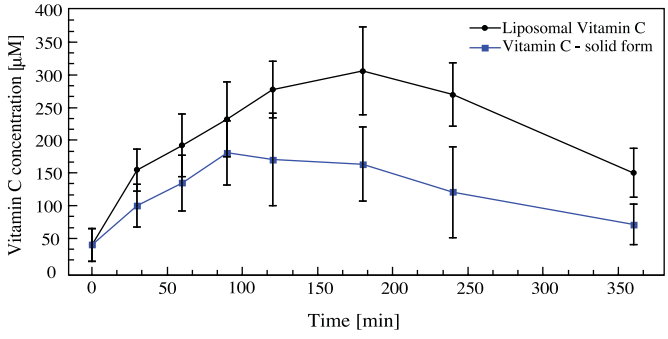

Figure 5. Averaged concentration profiles of sodium ascorbate in serum determined for two groups of persons, following the oral intake of 10 g of sodium ascorbate in the form of the aqueous solution (squares) and encapsulated in liposomes (circles). The lines between points were drawn arbitrarily to guide the eye. Values of plasma concentrations measured for specific time points for liposomal and non-liposomal dosages are significantly different ($p < 0.05$) for three last points (180, 240, and 360 min).

frequent dosing. Maintaining high levels of vitamin C in the digestive tract is dependent mainly on the rate of its hydrolysis. The vitamin C degradation can be significantly reduced by its association with lipid interfaces, which are abundant in the liposomal formulation (Wechtersbach *et al.* 2012). The effectiveness of the liposomal formulation depends, in addition to vitamin C content, on quality of liposomes quantitated by their size distributions (Ensign *et al.* 2012, Beilstein *et al.* 2016). The presented method of liposome containing vitamin C preparation produces a single population of vesicles, as demonstrated using DLS and electron microscopy techniques (Figure 1). The formulation is stable in time with respect to both, chemical composition and physicochemical properties, as required for any marketed product. When evaluating the formulation efficacy, it has been shown that lipid vesicles do not interfere with the vitamin C accessibility to cells, regardless of their type (Figure 2). This implies that, if a certain concentration of ascorbic acid in digestive tract is maintained for a sufficient time period, the high absorption level will be achieved and maintained. The other important aspect of the liposome formulation is possibility to deliver large doses of vitamin C for an extended period of time, since the lipid capsule mitigates the irritation of gastrointestinal tract typically accompanying large oral doses of ascorbate. In addition, as described elsewhere lipids by themselves have beneficial physiological effects (Davis *et al.* 2016). Subsequently, the formulation bioavailability has been demonstrated in the medical experiment on healthy volunteers, where liposomal vitamin C outperforms the traditional form with the respect to the maximal concentration as well as the halftime in serum (Figure 3). The other important feature of the presented formulation is that the process of liposome formation does not require toxic organic solvents. Pharmacologically acceptable glycerine has been used instead.

In summary, encapsulation of vitamin C in new types of liposomes causes the enhancement of vitamin C bioavailability on a physiological level, without compromising its potency on the cellular level. The liposomal formulation of vitamin C, in addition to its high activity, as ensured by elevated bioavailability, should also satisfy strict regulatory requirements regarding the content of potentially harmful compounds, stability and reproducibility of production processes. In the paper, it has been demonstrated that the new liposomal preparation can be produced with consistently stable critical parameters such as: chemical composition and homogeneity of the liposome population. In addition, the glycerine-based production process overcomes the major obstacle, common in other production processes, the need for application of pharmacological undesirable organic solvents such as ethanol.

Disclosure statement

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Data availability

The data that support the findings of this study are available from the corresponding author, PD, upon reasonable request.

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