An in-depth analysis of Lipolife® Liposomal Vitamin C

Research conducted by an Independent Laboratory in November 2016

SYNOPSIS

Ascorbic acid (vitamin C) is commonly used as an antioxidant. An antioxidant is a compound that neutralises free radicals and oxidants. When produced excessively, such as during certain diseases and physical exercise, free radical and oxidants may damage cells. Free radicals and oxidants are abundantly produced during inflammation, which lies at the basis of many diseases that include autoimmune diseases (e.g., rheumatoid arthritis, Crohn's disease), infections, and cancer. Cancer can develop as a result of chronic inflammation, as is for example the case in cirrhotic livers as a result of long-term alcohol abuse. Radicals are also produced in several neurodegenerative diseases such as Alzheimer's and Parkinson's.

Although free radicals and oxidants fulfil numerous important biological functions, their excessive production is deleterious to cell viability and overall health. Our body is capable of neutralising most free radicals and oxidants but could use some help during disease or strenuous physical activity. Ascorbic acid as a supplement could be used to help remediate the excessive production of free radicals and oxidants.

Vitamin C liposomes from Lipolife® were characterised to determine the size of the liposomes, size distribution and long-term stability under different storage conditions. The formulation was tested in a number of chemical and biological assays that were attuned to the intended use of the product and the abovementioned role of free radicals in (patho)physiology. The assays focused on the neutralisation of free radicals and oxidants, the capacity to kill cancer cells and the capacity to kill bacteria.

Based on the results, the following conclusions can be drawn with respect to Lipolife's® vitamin C liposomes.

1. The ascorbic acid:phospholipid molar ratio is 6.4;
2. The liposomes have a diameter of ~200 nm;
3. The size of the liposomes is homogeneous, particularly at (near-) neutral pH;
4. Liposome size is minimally affected by acidic pH in the short term;
5. The liposomes are stable at 4 °C, room temperature and body temperature (37 °C) for at least 36 days in terms of size and size distribution, regardless of the atmosphere (oxygen-rich versus oxygen-poor);
6. The vitamin C liposomes neutralise free radicals and oxidants;
7. The antioxidant capacity is marginally reduced after 36 day storage at room temperature and moderately reduced after storage at 37 °C. Storage at 4 °C fully preserves the antioxidative capacity of the formulation for at least 36 days. Accordingly, the product should preferably be stored under an oxygen-poor (e.g., nitrogen-rich) atmosphere;
8. The vitamin C liposomes kill cultured cancer cells derived from human bile duct tumours;
9. The vitamin C liposomes kill and/or arrest the growth of some types of cultured bacteria.

Data Set 1

Aim
To determine the phospholipid and ascorbic acid concentration in the product. This information serves to confirm the product content as specified on the label. The information may also be used to assess loss of the main compounds during the production process, when compared to known amounts of starting raw materials. Finally, the data can be used to determine encapsulation efficiency and to provide an indication of how much of the ascorbic acid is inside and outside the liposomes.

Main results
Mean ± SD ascorbic acid content: 995 ± 64 µM
Mean ± SD phospholipid content: 155 ± 38 µM
Ascorbic acid:phospholipid mole ratio: 6.42:1.00
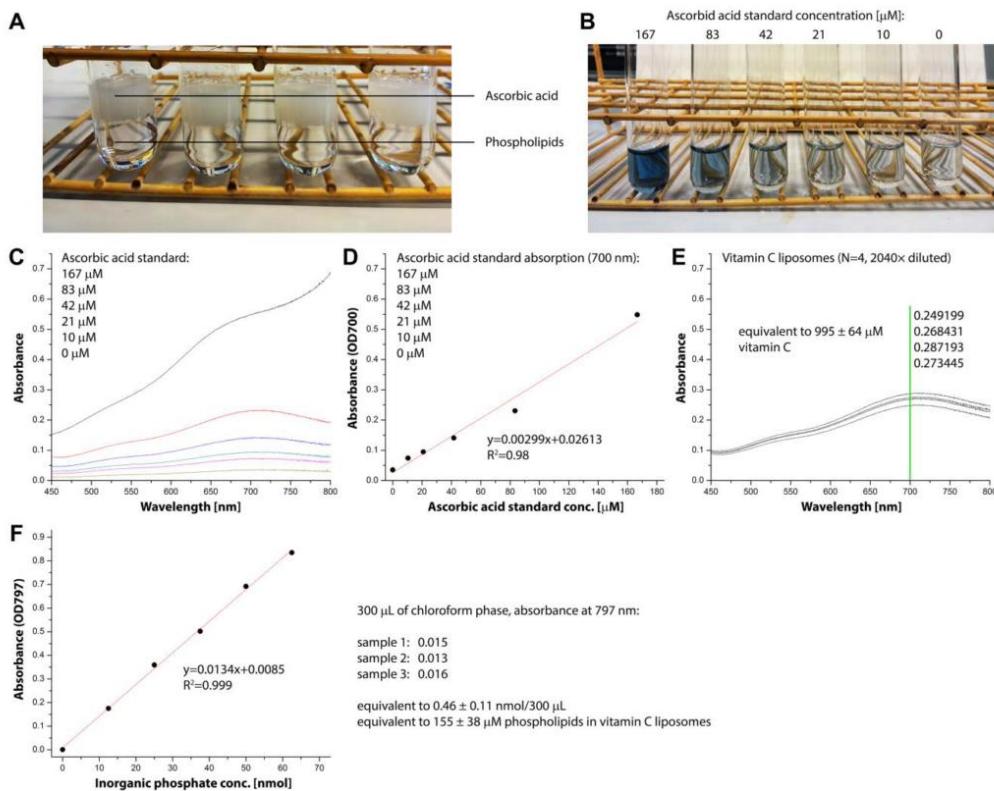
Figure 1. (**A**) Image of the liquid two-phase separation to extract ascorbic acid (top layer) and phospholipids (bottom layer) from the vitamin C liposomes. The content of ascorbic acid and phospholipids was subsequently determined in the respective phase using customised analytical assays, which entail chemical derivatization of ascorbic acid and phosphate. (**B**) Colour development of the ascorbic acid standards following chemical derivatisation. The standards were used to calculate the unknown ascorbic acid content in the product. The absorption spectra of the samples are provided in (**C**) and the absorbance value at 700 nm (OD700) is plotted for every standard concentration in (**D**). The linear fit function and goodness of fit (R^2) value are provided for the standard curve, on the basis of which the ascorbic acid concentration in the product was calculated.

(**E**) Absorption spectra of the chemically derivatised ascorbic acid taken from the top phase in (**A**). Analysis was performed on quadruplicate samples (i.e., N = 4). The absorbance at 700 nm (green line) is listed for every sample. These values can be plotted in the linear fit function (**D**, as the y-value) to extrapolate the ascorbic acid concentration (x-value), taking into account a dilution factor of 2,040. Calculations result in a mean ± standard deviation (SD) ascorbic acid concentration of 995 ± 64 µM in the product. (**F**) A same standard curve was prepared to determine the phospholipid content in the bottom phase (**A**). Samples were assayed in triplicate (N = 3) and the phospholipid content was calculated from the linear fit function, taking into account a sample volume of 300 µL. Calculations resulted in a mean ± SD phospholipid concentration of 155 ± 38 µM in the product. Abbreviations: nm, nanometer; µM, micromolar; nmol, nanomoles.

Methods and instructions on data interpretation

The Bligh & Dyer method was used to separate ascorbic acid from phospholipids in a 2phase extraction system. A customised assay was used to determine ascorbic acid concentration. This assay, as well as the assay used to determine phospholipid content, relies on chemical derivatisation of ascorbic acid and phospholipid-derived phosphate to a chromophore (a pigment, or coloured molecule). The consequent coloured product was measured spectrophotometrically at 700 nm. The ascorbic acid and phospholipid content in the product were calculated from the standard curve prepared with standards of known ascorbic acid and phosphate concentration.

The spectra of the pure ascorbic acid standards are presented to demonstrate that the assay, developed strictly for determining the ascorbic acid concentration in the product, is valid. The validity of the method is confirmed by the fact that the shape of the absorption spectrum of the standards is identical to the spectrum of the product-derived ascorbic acid. It can be concluded that any other chemicals that may be present in the ascorbic acid phase (Figure 1A, top layer) did not interfere with the assay, and therefore did not skew the results.

The molecular weight of ascorbic acid is 176.12 g/mol (grams per mole). This information can be used to calculate the amount (in g or kg) of ascorbic acid in the product for any given volume. Please note that 1 M = 1 mol/L (moles per liter), that 1 mM = 1 millimol/L, that 1 µM = 1 micromol/L, etc.

We did not determine which phospholipid species are present in the product and therefore, cannot provide a molecular weight to calculate the phospholipid content (in g or kg) of the product. A 6.42 molar ratio was determined, implying that for every phospholipid molecule in the formulation there are 6.42 ascorbic acid molecules.

Data Set 2

Aim

To determine the diameter of the particles (liposomes) and the size distribution (polydispersity index), which indicates whether the particles are homogeneous or heterogeneous in size. Because the liposomes end up in the stomach after oral intake, where the pH is ~1, the size and polydispersity index of the liposomes were also determined in a pH range of 1 (acidic) to 7.4 (neutral).

Main results

The liposomes have a mean diameter of ~200 nm in a pH range of 2.00-7.38.

At pH = 1.02, the diameter was around 250 nm (Figure 2A), suggesting that the liposomes became unstable and started aggregating. The polydispersity index of the liposomes in neutral PBS buffer was around 0.2, indicating that the size of the liposomes was relatively homogeneous (Figure 2B, red line). At lower pH, especially at pH ≤ 2.00, the polydispersity index increased (Figure 2B), suggesting that the liposomes were destabilized by the highly acidic conditions quite rapidly after exposure (within 2.5 hours). Representative size distributions are provided per pH class in Figure 2C-I.

Methods and instructions on data interpretation

Particle size and polydispersity index were measured by photon correlation spectroscopy within 2.5 hours after addition to PBS at different pH. Five measurements of 10 iterations were performed per sample. The particle diameter is given in nanometer (nm). The polydispersity index is given in arbitrary units and lies between 0.000 and 1.000, where a value of 0.000 corresponds to a particle population in which all particles have the same diameter (i.e., homogeneous particle size; narrow distribution histogram), whereas a value of 1.000 corresponds to a particle population comprised of particles of many different diameters (i.e., heterogeneous particle size; broad distribution histogram). A polydispersity index of ≤ 0.200 is considered to reflect a particle population with a homogeneously distributed particle diameter.
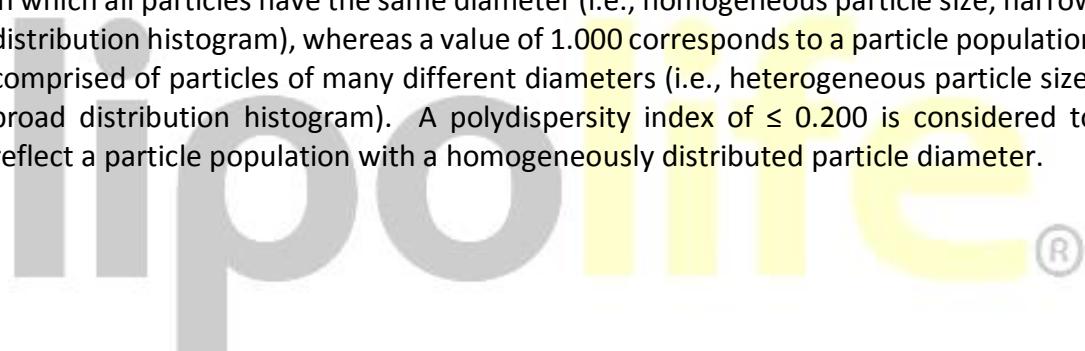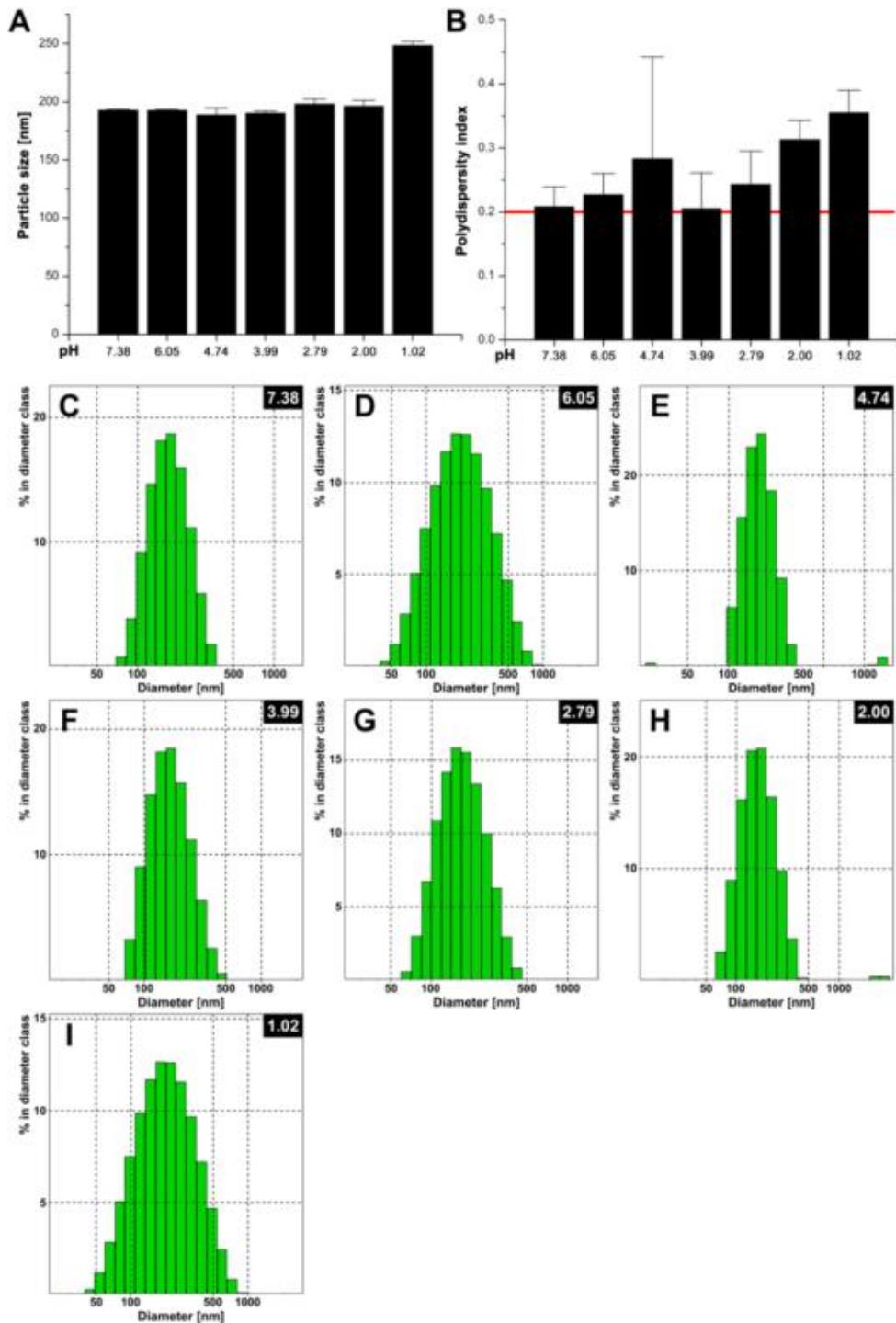
Figure 2. (**A**) Mean ± standard deviation (SD) liposome diameter, plotted as a function of pH (xaxis). (**B**) Mean ± SD liposome polydispersity index, plotted as a function of pH (x-axis). (**C-I**) Representative size distribution histograms for every pH class, indicated in the upper right corner.

Data Set 3

Aim
To determine the extent to which the vitamin C liposomes can scavenge radicals. This aim corresponds to the main intended use of the product.

Main results
The vitamin C liposomes were efficient in scavenging free electrons derived from the transition metal ferrous iron [Fe(II)] (Figure 3B) as well as hydroxyl radicals generated by the Fenton reaction (Figure 3D), which produces the most cytotoxic and reactive radical species, namely the hydroxyl radical. Both reactions are physiologically relevant in disease or during strenuous exercise. The antioxidative capacity was measured with a custom-made fluorogenic probe that is intrinsically non-fluorescent. Oxidation of the probe by free electrons and radicals causes the probe to become highly fluorescent. An effective antioxidant will therefore reduce the degree of fluorescence emitted by the probe when exposed to free electrons and radicals, as illustrated in Figure 3A for the electron-donating transition metal Fe(II) and in Figure 3C for the Fenton reaction-generated hydroxyl radical.

The scavenging occurred in an ascorbic acid concentration- and phospholipid concentration-dependent manner, meaning that higher concentrations of ascorbic acid and phospholipids scavenged free electrons and radicals to a greater extent. The final concentrations of product tested were 3.33% volume/volume (corresponding to 33 µM ascorbic acid and 5 µM phospholipids, * in Figure 3B and D), 0.33% volume/volume (corresponding to 3.3 µM ascorbic acid and 0.5 µM phospholipids,*), 0.033% volume/volume (corresponding to 0.33 µM ascorbic acid and 0.05 µM phospholipids, *), and 0.0033% volume/volume (corresponding to 0.033 µM ascorbic acid and 0.005 µM phospholipids, *), based on the characterization in Data Set 1.

Methods and instructions on data interpretation

The production of reactive transients was mediated by the transition metal Fe(II), which donates a free electron for ultimate oxidation of a substrate (the molecule that becomes oxidized). In this case the substrate was DCFH2, which is a fluorogenic probe. A fluorogenic probe is a probe that does not emit any fluorescence in native state until it undergoes chemical modification, such as by oxidation. In a second test arm, hydroxyl radicals were produced through the Fenton reaction. Both test systems were optimized such that the probe produced abundant fluorescence upon oxidation while significant antioxidant effects were clearly detectable.
All experiments were performed in triplicate.

When antioxidants such as the vitamin C liposomes are added to the reaction mixture, the electrons that would normally lead to oxidation of the probe and thus fluorescence, are captured by the antioxidant. An antioxidant therefore reduces the fluorescence of the probe, whereby a reduction in fluorescence is by definition indicative of the antioxidative capacity of the antioxidant. The more the fluorescence is reduced, the more potent the antioxidant.

Naturally, the concentration of the antioxidant is important, as the presence of more antioxidant molecules increases the likelihood that an electron is captured.

It should also be noted that an antioxidant effect may stem from both the ascorbic acid as well as the phospholipids that make up the liposomes. It should further be noted that hydroxyl radicals attack basically any (bio)molecule in their direct vicinity. The assays with Fe(II) are therefore more representative of the true antioxidative capacity of the vitamin C liposomes.
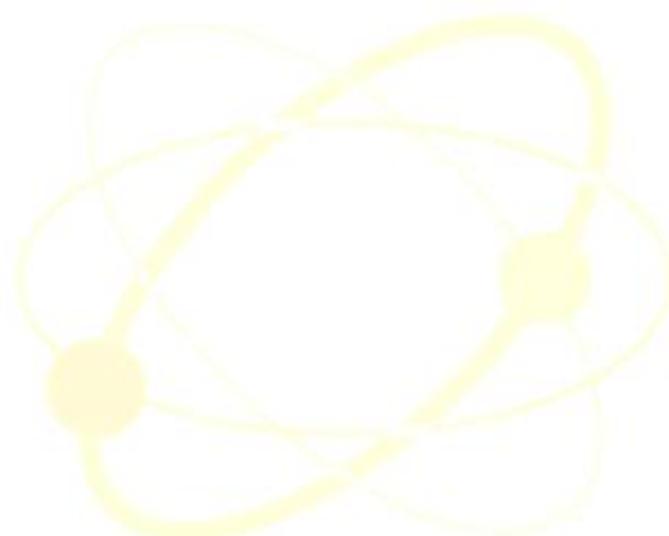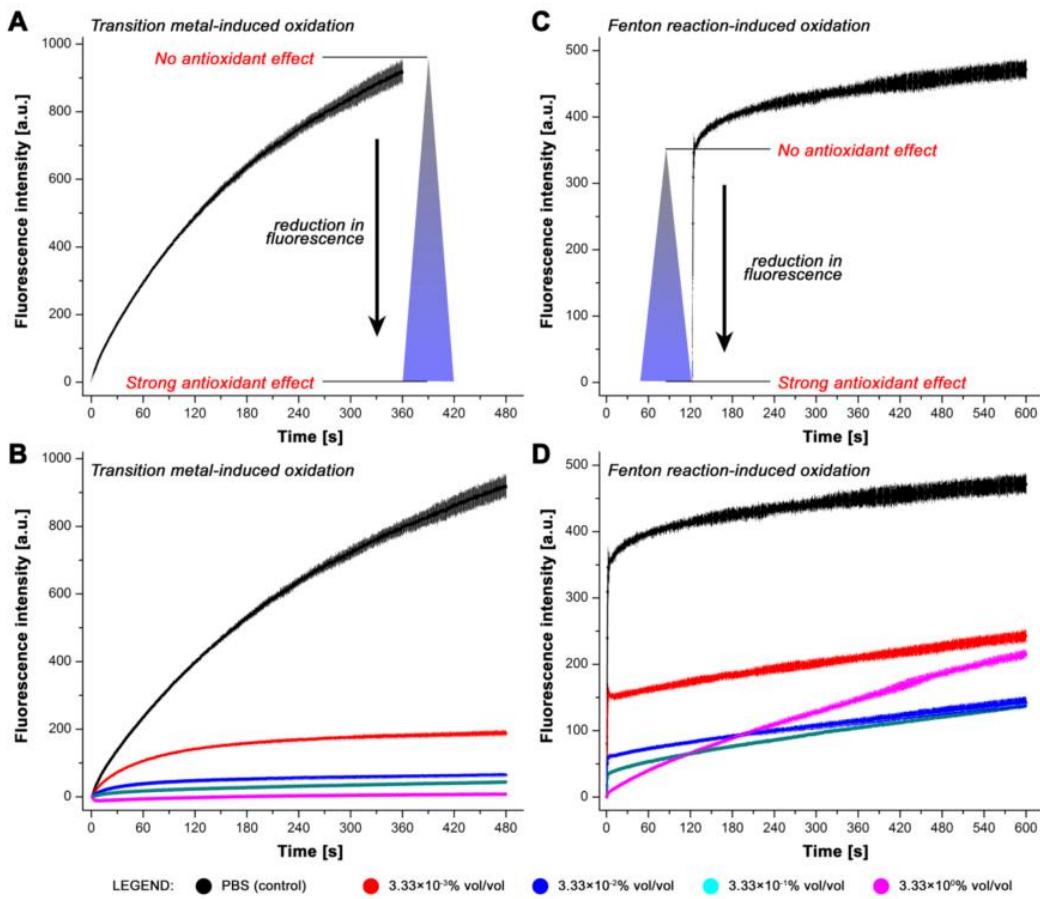
Figure 3. Antioxidative capacity of vitamin C liposomes, measured by the potential of the product to inhibit the free electron- and radical-induced oxidation of the fluorogenic probe DCFH2. The inhibition of probe oxidation by antioxidants results in reduced fluorescence emission intensity, illustrated schematically in (**A**) and (**C**) for the transition metal Fe(II)-induced oxidation and the Fenton reaction induced oxidation, respectively. The black trace in (**A**) and (**C**) represents the evolution of fluorescence (i.e., probe oxidation) under circumstances where no antioxidant is present. These circumstances represent control conditions. Note that the Fe(II)-derived electron reacts at a much slower rate with the probe than the Fenton reaction-produced hydroxyl radical, which is the most reactive radical of all radical species. When an antioxidant is added, the (radical) electrons responsible for oxidation are captured by the antioxidant and therefore become unable to oxidize the probe, causing a reduction in fluorescence. This effect was demonstrated for the product in case of the Fe(II)-induced oxidation reaction (**B**) and the Fenton-induced oxidation reaction (**D**) in a vitamin C liposome concentration-dependent manner (see legend at the bottom of figure). Data are plotted as mean ± standard deviation for N = 3 per group.

Abbreviations: a.u., arbitrary units; s, seconds; PBS, phosphate buffered saline; vol., volume.

Data Set 4

Aim

To determine the stability of the vitamin C liposomes during storage for 36 days at different temperatures and under an oxygen-rich and oxygen-poor atmosphere.

In this data set, liposome size and polydispersity index were used as measure of stability. These experiments were conducted to get an indication of shelf life and storage requirements of the product.

Main results

Liposome diameter did not differ considerably between batches of vitamin C liposomes stored for 36 days at 4 °C and at room temperature (~22 °C), regardless of whether the formulations were kept under an atmosphere composed of air or pure nitrogen gas (i.e., oxygen-poor atmosphere) (Figure 4A). Accordingly, there was no difference in liposome diameter relative to the freshly opened vitamin C liposomes (Figure 4A, black bar, taken from Data Set 1). Storage at 37 °C, equivalent to body temperature, caused a slight increase in liposome size irrespective of the type of atmosphere. The polydispersity index ranged from 0.2 to 0.3, indicating that the liposomes did not undergo a considerable change in the distribution of liposome diameter over time.

In conclusion, the best storage temperature of the vitamin C liposomes is ≤ 22 °C on the basis of the physical properties of the liposomes. At these temperatures, the liposomes do not seem to behave in a manner that suggests instability.

Methods and instructions on data interpretation

Upon opening of the product, aliquots (small portions of the whole product volume) were transferred to 15-mL tubes, of which half was purged with nitrogen gas and the other half was left in an atmosphere composed of air. The tubes were sealed with parafilm to prevent gas exchange. In the non-purged group, one tube was stored at 4 °C, one tube was stored at room temperature, and one tube was placed in an incubator maintained at 37 °C. All aliquots were stored in the dark. The nitrogen-purged aliquots were allocated in the same manner.

After 36 days of storage, the aliquots were assayed by photon correlation spectroscopy as described in Data Set 1. The vitamin C liposomes were suspended in PBS, pH = 7.4.
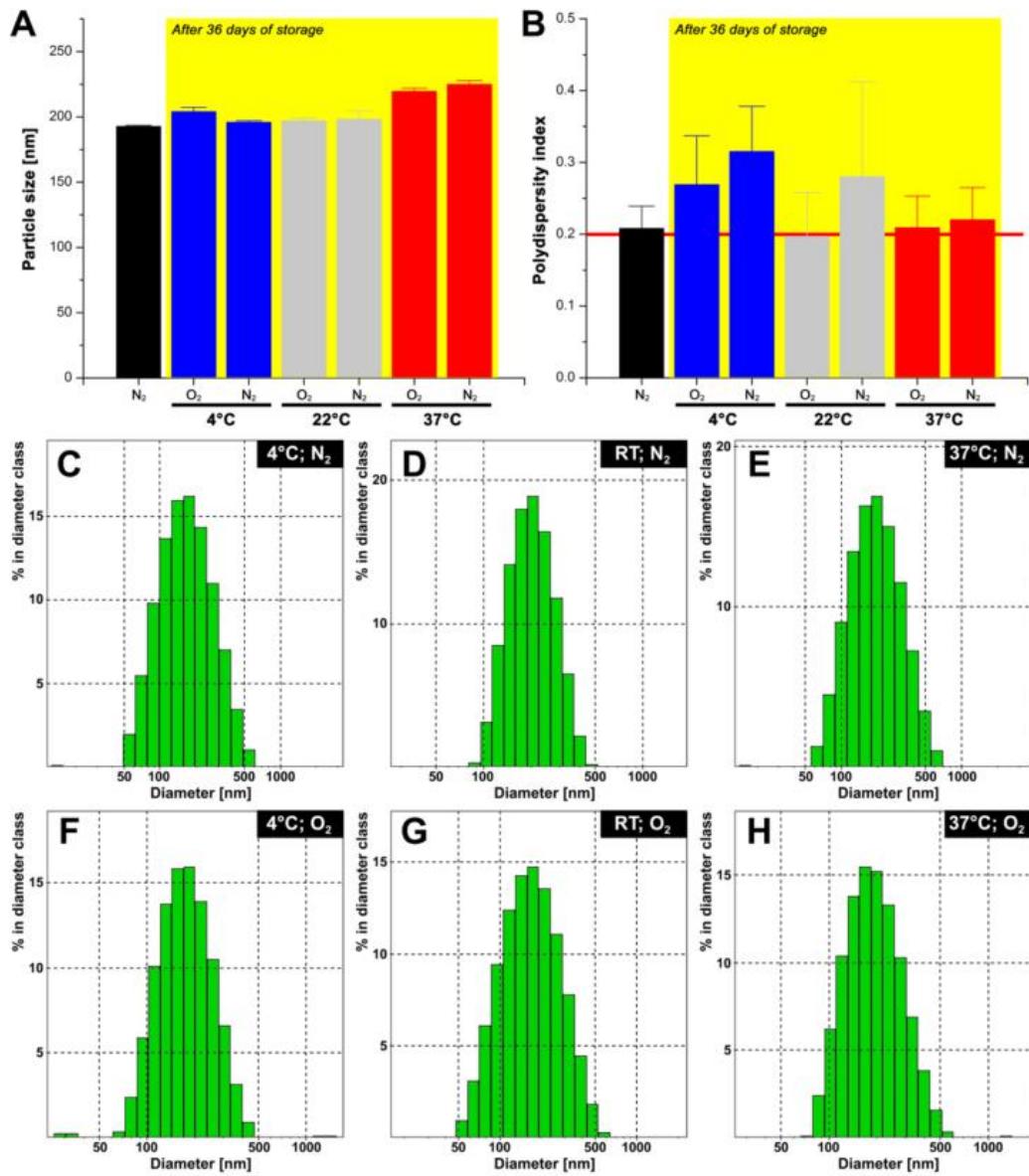
Figure 4. (**A**) Mean ± standard deviation (SD) liposome diameter, plotted as a function of storage conditions (x-axis). (**B**) Mean ± SD liposome polydispersity index, plotted as a function of storage conditions (x-axis). The black bars in (**A**) and (**B**) represent the values of fresh product in PBS at pH = 7.4. (C-H) Representative size distribution histograms for every storage condition, indicated in the upper right corner. Abbreviations: nm, nanometer; N2, nitrogen gas; O2, oxygen; RT, room temperature.

Data Set 5

Aim

To determine the antioxidative capacity of vitamin C liposomes after storage for 36 days at different temperatures and under an oxygen-rich and oxygen-poor atmosphere. This data set served as a follow-up to Data Set 4 to determine whether the functional properties of the product had deteriorated after storage.

Main results

The antioxidative capacity of the stored aliquots was assayed by the same approach as used in Data Set 3. The Fe(II)-induced oxidation results are provided in the top row of Figure 5 for every storage temperature, whereas the Fenton-induced oxidation results are depicted in the bottom row of Figure 5 for every temperature. The data were compared to the antioxidative capacity of freshly opened vitamin C liposomes.

In regard to the Fe(II) experiments (Figure 5, top row), 36-day storage caused the antioxidative capacity of the vitamin C liposomes to deteriorate. The deterioration was mild at 4 °C and independent of the type of storage atmosphere, as evidenced by the proximity of the blue (storage under nitrogen) and red trace (storage under air) to the grey trace (antioxidative capacity of fresh vitamin C liposomes). The experiments performed at room temperature and at 37 °C revealed that (1) storage under nitrogen gas is better for the functionality of the product (less oxidation of the probe, so greater antioxidative capacity) compared to storage under an oxygen-rich atmosphere, and (2) storage at temperatures of > 4 °C reduced the antioxidative capacity of the product in time in a temperature-dependent manner.

These data were mirrored by the Fenton reaction experiments (Figure 5, bottom row), albeit to a lesser extent due to the fact that the generated hydroxyl radicals attack basically any molecule. Consequently, for this specific reaction, differences in the concentration of antioxidants (Data Set 3) are expected to be more pronounced than when the experiments are performed at equal vitamin C liposome concentration but with potentially oxidized product contents (e.g., vitamin C liposomes stored at 37 °C).

Methods and instructions on data interpretation

The preparation of samples for storage is described in Data Set 4. The experimental approach is described in detail in Data Set 3. The experiments were performed at a vitamin C liposome concentration of 0.0033% volume/volume, corresponding to 0.033 µM ascorbic acid and 0.005 µM phospholipids. To generate the grey curve in Figure 5, the fluorescence intensity of the vitamin C liposomes in Figure 3 (0.0033% volume/volume) was divided by the fluorescence intensity of the PBS control in Figure 3 for every time point. The fraction at every time point was multiplied by the respective fluorescence intensity of the PBS control in Figure 5. This extrapolation represents the hypothetical antioxidative capacity of fresh vitamin C liposomes in the current experimental setup (which was performed 36 days later with the same product batch).
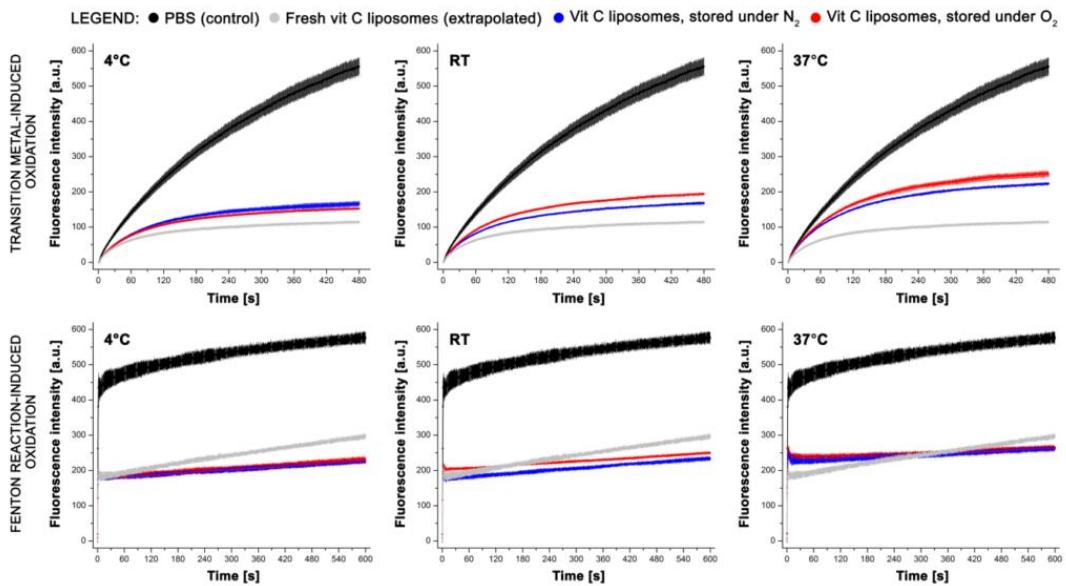
Figure 5. Antioxidative capacity of vitamin C liposomes that had been stored for 36 days under anitrogen or oxygen atmosphere at 4 °C (left column), room temperature (central column), and 37 °C (right column), indicated in the upper left corner.

The antioxidative capacity was assayed in the Fe(II)-induced oxidation reaction (top row) and the Fenton-induced oxidation reaction (bottom row). Every experimental group is described in the legend provided at the top of the figure. Experiments were performed at a vitamin C liposome concentration of 0.0033% volume/volume, corresponding to 0.033 µM ascorbic acid and 0.005 µM phospholipids.

Experiments were performed in triplicate per group.

Data are plotted as mean ± standard deviation.

Abbreviations: PBS, phosphate buffered saline; vit, vitamin; N2, nitrogen gas; O2, oxygen; a.u., arbitrary units; s, seconds; RT, room temperature.
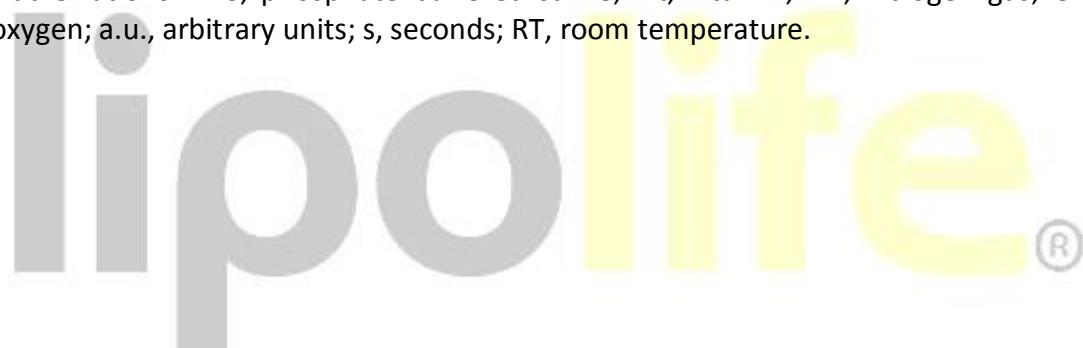
Data Set 6

Aim

To determine whether the vitamin C liposomes are toxic to cancer cells derived from human bile duct tumours and cells of the innate immune system (macrophages). Toxicity was first measured by determining mitochondrial function following 3-day incubation of cultured cells with increasing concentrations of vitamin C liposomes.

Main results

Human bile duct cancer cells (cell lines Sk-Cha1, Mz-Cha1, Mz-Cha2, and TFK-1) exhibited impaired mitochondrial activity following incubation with the vitamin C liposomes. At the lowest vitamin C liposome concentration (0.63% volume/volume, corresponding to 6.3 µM ascorbic acid 1.0 µM phospholipid concentration), Sk-Cha1 cells and Mz-Cha1 cells had no mitochondrial metabolic activity (Figure 6).
Mz-Cha2 and TFK-1 cells lost mitochondrial metabolic activity at a vitamin C liposome concentration of 2.5% (24.9 µM ascorbic acid 3.9 µM phospholipids) and 5.0% (49.8 µM ascorbic acid, 7.8 µM phospholipids), respectively.

Mitochondria are responsible for producing energy (ATP) to sustain cell metabolism. Loss of mitochondrial activity therefore leads to a loss of energy, ultimately causing the cells to die. The loss of mitochondrial activity is an early sign of impending cell death. The differential response to vitamin C liposomes may be due to the differential metabolic rate of the cells, where cells with higher metabolic activity are more susceptible to pharmaceutical agents (such as vitamin C liposomes) than cells with lower metabolic activity.

Cultured mouse macrophages (RAW 264.7 cells) were also susceptible to the vitamin C liposomes, exhibiting complete loss of mitochondrial metabolic activity at a vitamin C liposome concentration of 1.25% (12.4 µM ascorbic acid and 1.9 µM phospholipids) (Figure 6). It should be noted that the macrophages have a 'cancer phenotype' in that they have been immortalised. They also have a high metabolic rate. Loss of mitochondrial metabolic activity does not mean that circulating macrophages in vivo will de facto undergo the same fate if they encounter vitamin C liposomes.
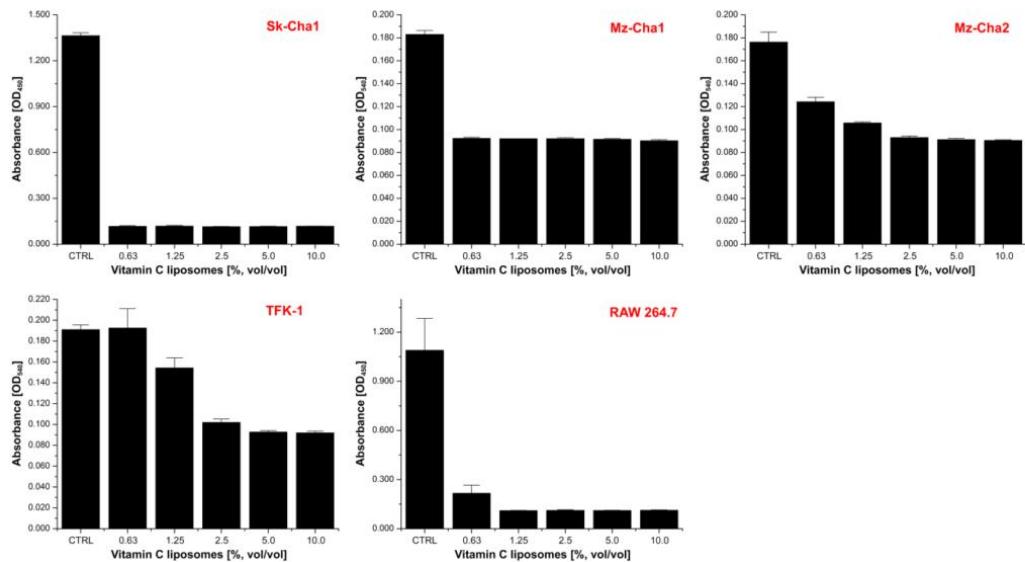
Figure 6. Changes in mitochondrial metabolic activity in cultured human bile duct cancer cells and mouse macrophages (cell lines indicated in the top right corner) after 3-day incubation with increasing concentrations of vitamin C liposomes (x-axis). Phosphate buffered saline was used as control.

Mitochondrial activity was assayed with WST-1, which is converted to a chromophore by metabolically active mitochondria that can be measured spectrophotometrically. The mean ± standard deviation absorbance at the indicated wavelength is plotted (y-axis) as a measure of mitochondrial metabolic activity for N = 4 per group. Note that the absorbance at which the bars plateau corresponds to background absorption that does not signify metabolic activity.

Abbreviations: ODxxx, optical density at wavelength xxx; CTRL, control; vol, volume.

Methods and instructions on data interpretation

Cytotoxicity of vitamin C liposomes in different human cancer cell lines (Mz-Cha1, Mz-Cha2, Sk-Cha1, TFK-1) and mouse macrophages (RAW 264.7) was determined as a function of concentration. Using multiple cell lines derived from 1 type of cancer provides a more representative indication of the cancer-killing efficacy of the tested product. The macrophages were included as test system to get an indication of how cells of the innate immune system respond to the product. Cells were incubated with vitamin C liposomes for 3 days under standard culture conditions.

The outcome parameter was mitochondrial metabolic activity (WST-1 assay), which was measured spectrophotometrically. A high absorbance means that the mitochondria in cells are metabolically active. A low absorbance means that mitochondria have lost metabolic activity, which is a sign of cytotoxicity of the added product. An x-axis plateau implies that the cells are no longer viable (the residual absorbance is due to background).

Data Set 7

Aim

To corroborate the vitamin C liposomes toxicity data in Data Set 6 in cancer cells derived from human bile duct tumours and cells of the innate immune system (macrophages). Toxicity was determined by total protein content in the culture plate following 3-day incubation of cultured cells with increasing concentrations of vitamin C liposomes.

Main results

The protein data (Figure 7) confirm the mitochondrial metabolic activity data in Figure 6. Whereas loss of mitochondrial metabolic activity is considered an early stage of cell death, total protein content reflects fully executed cell death. Human bile duct cancer cells (SkCha1, Mz-Cha1, Mz-Cha2, and TFK-1) all died following incubation with the vitamin C liposomes, although in a vitamin C liposome concentration-dependent manner that was dependent on cell type. At the lowest vitamin C liposome concentration (0.63% volume/volume, corresponding to 6.3 µM ascorbic acid 1.0 µM phospholipid concentration), Sk-Cha1 cells and Mz-Cha1 cells were no longer viable (Figure 7). The viability of Mz-Cha2 and TFK-1 cells was completely lost at a vitamin C liposome concentration of 5.0% (49.8 µM ascorbic acid, 7.8 µM phospholipids). Cultured mouse macrophages (RAW 264.7 cells) exhibited complete loss of viability at a vitamin C liposome concentration of 0.63% (6.3 µM ascorbic acid 1.0 µM phospholipid concentration) (Figure 7). The same cautionary note on data interpretation applies here as was provided in Data Set 6.
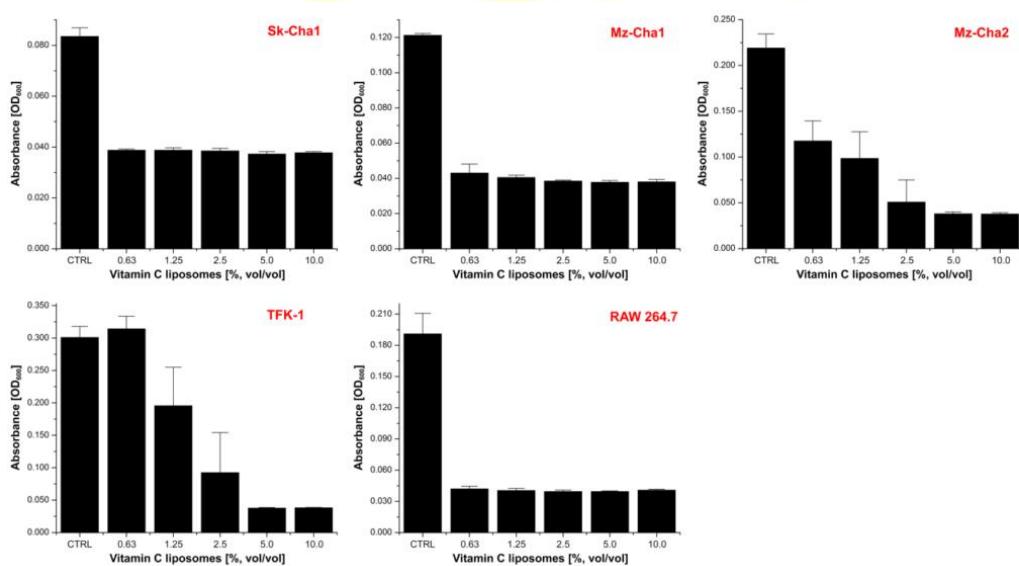

Figure 7. Changes in cell viability in cultured human bile duct cancer cells and mouse macrophages (cell lines indicated in the top right corner) after 3-day incubation with increasing concentrations of vitamin C liposomes (x-axis). Phosphate buffered saline was used as control.

Total protein content was assayed with sulforhodamine B, which binds to viable cells still attached to the culture dish, that was measured spectrophotometrically. The mean ± standard deviation absorbance at the indicated wavelength is plotted (y-axis) as a measure of cell viability for N = 4 per group.

Note that the absorbance at which the bars plateau corresponds to background absorption that does not signify residual viability. Abbreviations: ODxxx, optical density at wavelength xxx; CTRL, control; vol, volume.

Methods and instructions on data interpretation

Cytotoxicity of vitamin C liposomes in different human cancer cell lines (Mz-Cha1, Mz-Cha2, Sk-Cha1, TFK-1) and mouse macrophages (RAW 264.7) was determined as a function of concentration. Cells were incubated with vitamin C liposomes for 3 days under standard culture conditions. The outcome parameter was cell viability (sulforhodamine B assay), which was measured spectrophotometrically. A high absorbance means that the cells are viable. The staining of adherent cancer cells is what accounts for the absorbance. A low absorbance means that the cells have detached from the culture plate (which dead cells do) and are therefore not stained by the sulforhodamine B. This is a sign of cytotoxicity imparted by the added product. An x-axis plateau implies that the cells are no longer viable (the residual absorbance is due to background.)

Data Set 8

Aim
To determine whether preconditioning of cells with vitamin C liposomes protects the cells from oxidative stress induced by hydrogen peroxide, an oxidant that can damage cells when produced excessively.

Main results
Human bile duct cancer cells (Sk-Cha1) and mouse macrophages were pre-treated with vitamin C liposomes for 1 hour at the final concentrations listed in Figure 8 and subjected to oxidative stress by 24-hour incubation with hydrogen peroxide. The WST-1 assay was used after 24-hour incubation to determine mitochondrial metabolic activity (Data Set 6) and the sulforhodamine B assay was used as a measure of cell viability (Data Set 7).

In Sk-Cha1 cells, the lethality of the vitamin C liposomes was too extensive to study any protective (antioxidant) effects, given the extreme loss of mitochondrial metabolic activity and viability at the lowest tested concentration (0.63% volume/volume, corresponding to 6.3 µM ascorbic acid 1.0 µM phospholipid concentration).

In RAW 264.7 macrophages, the anticipated effect was observed to a mild extent at the 0.63% and 1.25% vitamin C liposome concentration, as evidenced by the increase in WST-1 and sulforhodamine B absorbance. However, at concentrations of > 1.25%, the cytotoxicity started masking the cytoprotective effects of the vitamin C liposome preconditioning. These data signify increased mitochondrial metabolic activity and viability and thus protection against hydrogen peroxide-induced cell death.

In both instances the assay must be optimised in terms of vitamin C liposome incubation time, final vitamin C liposome concentration, and hydrogen peroxide content. A protective effect seems to be present and warrants further investigation.

Methods and instructions on data interpretation

Sk-Cha1 cells and RAW264.7 macrophages were incubated for 1 hour with vitamin C liposomes. This step ensured that the product entered the cells where it would protect the cells from hydrogen peroxide-induced oxidative stress. Oxidative stress from excessively produced hydrogen peroxide is a hallmark event in cell physiology and pathophysiology, and is therefore clinically most relevant. Mitochondria produce hydrogen peroxide through superoxide, which is converted to the less noxious hydrogen peroxide by a mitochondrial enzyme called superoxide dismutase. Hydrogen peroxide is able to pass through membranes and therefore oxidize biomolecules outside of mitochondria. Following priming of cells with the product, which was used to increase the cells' antioxidative capacity, hydrogen peroxide was added at a sub-lethal concentration (25 µM) to the cells. At this concentration, the cells will experience oxidative stress without massively dying. The cells were incubated for 24 hours to ensure that the oxidative stress was sufficiently persistent.

After 24-hour incubation, the cells were assayed with WST-1 to determine mitochondrial metabolic activity and sulforhodamine B to determine cell viability, as described in Data Sets 6 and 7, respectively. As in the previous data sets, high absorbance indicates that the cells contain metabolically active mitochondria and that the cells are viable. It was expected that the absorbance would increase in the hydrogen peroxide-subjected cells that were preconditioned with the vitamin C liposomes because the liposomes would protect the cells from lethal oxidative stress. The oxidative stress induced by hydrogen peroxide causes the absorption to decrease.
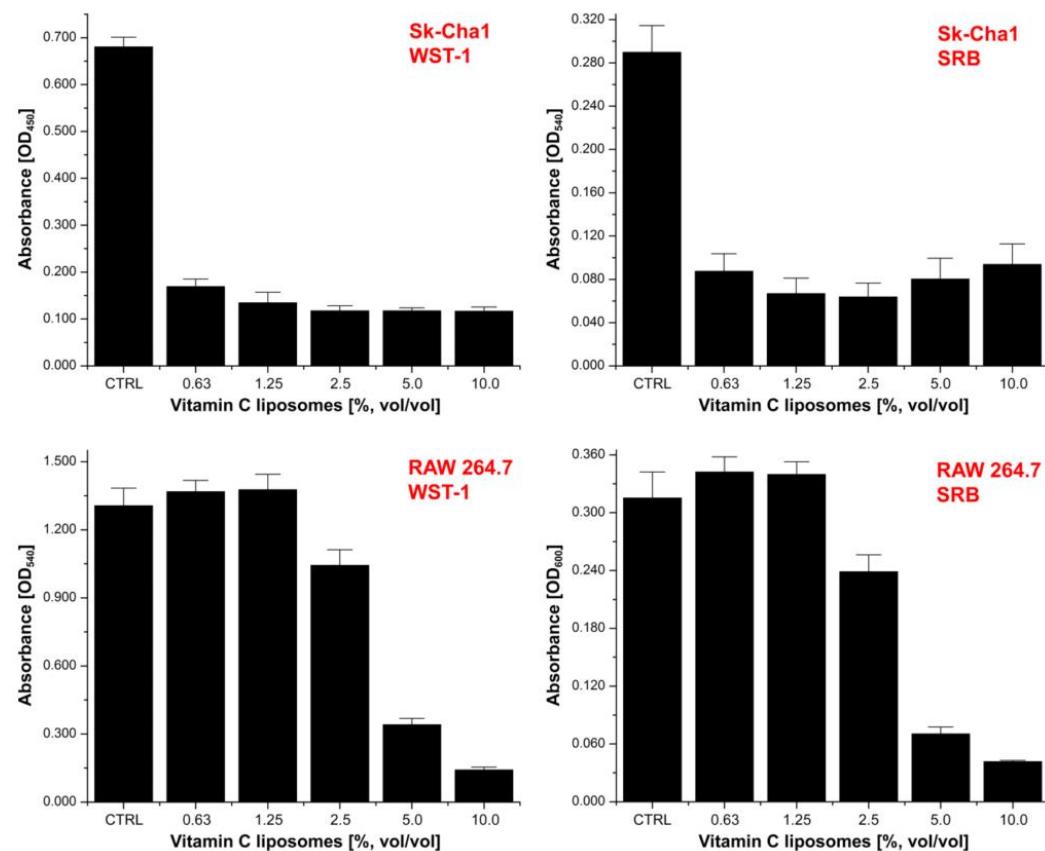

Figure 8. Cell mitochondrial metabolic activity (WST-1) and viability (sulforhodamine B) in cultured Sk-Cha1 cells and macrophages (cell lines indicated in the top right corner) after preconditioning with increasing concentrations of vitamin C liposomes (x-axis) and subsequent exposure to a sub-lethal dose of hydrogen peroxide to induce oxidative stress. Phosphate buffered saline was used as control. WST-1 (mitochondrial metabolic activity) and sulforhodamine B (cell viability) were determined spectrophotometrically. The mean ± standard deviation absorbance at the indicated wavelength is plotted (y-axis) for N = 4 per group.
Abbreviations: ODxxx, optical density at wavelength xxx; CTRL, control; vol, volume; SRB, sulforhodamine B.

Data Set 9

Aim

To determine whether the vitamin C liposomes are capable of killing bacteria. This is important to assess the breadth of indications for which the product could be used.

Main results

Two different bacterial species were used in the experiments: Escherichia coli (E. coli) and Staphylococcus simulans (S. simulans). E. coli is a common pathogen that is present in e.g., improperly cooked meats such as chicken and causes (severe) gastroenteritis. S. simulans is responsible for numerous animal diseases (rats, birds, cattle) and can cause infections of invasive medical devices. The bacteria were grown in agar and inoculated with increasing dosages of bactericidal antibiotics (kanamycin and ampicillin), ascorbic acid, and vitamin C liposomes, as indicated by the black 'x' in Figure 9. The dosing regimen table below the agar panels shows the concentrations used for each compound. Tests were performed in duplicate (left 2 panels are E. coli, right 2 panels are S. simulans). Kanamycin killed bacteria and/or retarded their growth in a concentration-independent manner, as evidenced by the similar size of the 'halos' with increasing concentration in both bacterial species. Ampicilin was only effective at the highest dose. Ascorbic acid had no effect on the bacteria, whereas the vitamin C liposomes exerted a bactericidal effect in S. simulans but not E. coli. It can therefore be concluded that the vitamin C liposomes are able to kill certain species of cultured bacteria.
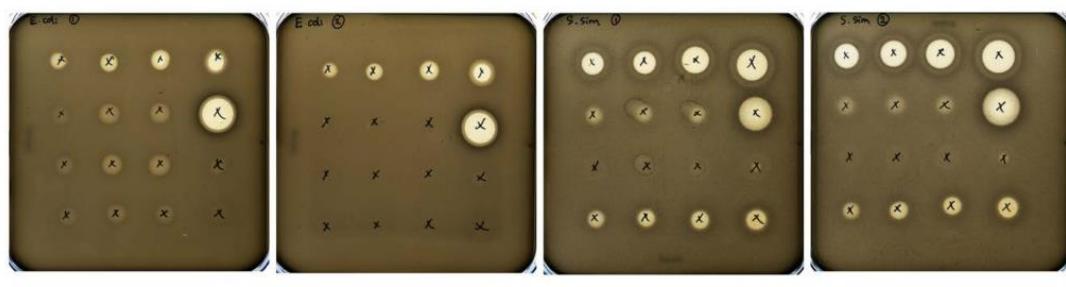

	0.17 µg	0.25 µg	0.50 µg	1.00 µg
Kanamycin	0.17 µg	0.25 µg	0.50 µg	1.00 µg
Ampicillin	0.33 µg	0.50 µg	1.00 µg	2.00 µg
Vitamin C	20 µg	40 µg	80 µg	160 µg
Vitamin C liposomes	50× dil.	30× dil.	20× dil.	10× dil.

Figure 9. Bactericidal effects of kanamycin, ampicillin, ascorbic acid, and vitamin C liposomes in Escherichia coli (left 2 panels) and Staphylococcus simulans (right 2 panels). The 'x' marks the spot of inoculation while the halo signifies the absence of bacteria (i.e., bacterial cell death).

Methods and instructions on data interpretation

Square petri dishes were filled with two layers of agar. The bottom layer was solid and served as base. The top layer was semi-solid and contained Escherichia coli and Staphylococcus simulans bacteria. Next, selective spots were inoculated with kanamycin (0.17-1.00 μg), ampicillin (0.33-2.00 μg), ascorbic acid (20-160 μg), and vitamin C liposomes (2-10% volume/volume). Kanamycin and ampicillin are antibiotics and hence served as positive control (intended to kill the bacteria). Ascorbic acid served as control for the vitamin C liposomes.

A 'halo' effect is observed when bacteria are killed and/or do not grow in the inoculated region because the region contains a bactericidal agent.

The whiter and larger the halo, the more potent the antibiotic effect.
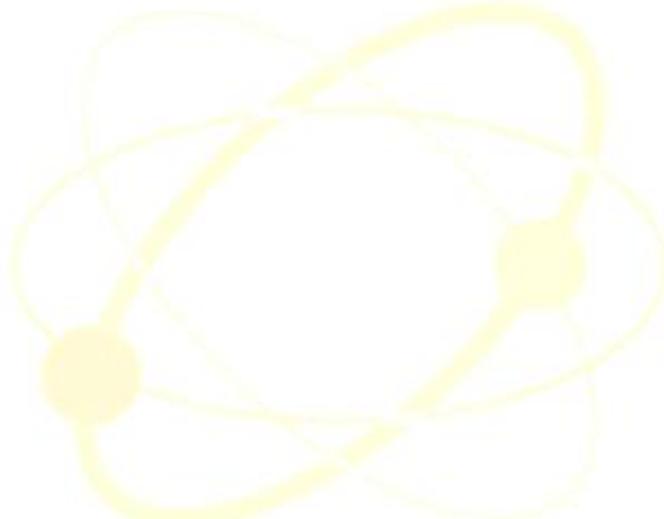
DISCLOSURE

The data should be interpreted in the context of the experimental setting and conditions. For instance, the vitamin C liposomes killed cultured cancer cells, but this does not mean that the product cures cancer or that it can be used to fight cancer in humans. Lipolife® will only openly support the claims and conclusions made in the synopsis and within the limits of the experimental setup.