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Research Article

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Efficacy and safety of sulforaphane-loaded emulsomes as tested on MCF7 and MCF10A cells

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Abstract

Objectives: Sulforaphane is well-known for its anti-cancer properties particularly against breast, skin and prostate cancers. High sensitivity of sulforaphane to oxygen, heat, and alkaline conditions, as well as its poor oral bioavailability and water instability limit its use in medicine. In this study, we aim to overcome the prementioned limitations by encapsulating sulforaphane within a lipid-based drug delivery system, known as emulsome, and investigate the anti-cancer features of the attained formulation.
Methods: The stability and dispersity of the formulation were assessed sequentially by zeta sizer, scanning electron microscopy and confocal laser scanning microscopy. Cell culture studies were performed to evaluate the anticancer activity of the formulation.
Results: Sulforaphane-loaded emulsomes with an average particle size of 246.0±14.1 nm, an average zeta potential of −23.5±2.4 mV and a polydispersity index of around 0.38 were produced. Encapsulations up to 0.036 mg/mL sulforaphane concentration was achieved. When MCF7 breast cancer cells were treated with sulforaphane-loaded emulsomes, a significant decrease was observed in proliferation of the cells along 72 h. In control group, emulsomes were found safe as tested at same concentrations on MCF-10a healthy cells. Applied as dissolved in DMSO, free sulforaphane with an IC_{50} value of 1.2 µM was more effective against MCF7 cells than sulforaphane-loaded emulsome formulation having a IC_{50} value 21.1 µM.
Conclusions: Sulforaphane-loaded emulsomes were obtained as stable, moderately disperse suspensions. Delivery of the bioactive compound into the cells were achieved. Yet, its biological activity remained behind its free form.

Keywords: sulforaphane; MCF7 breast cancer cell line; nanomedicine; lipid nanoparticles; limited bioavailability

Introduction

Drug candidates that are extracted from natural resources present a renewed attentiveness. Vincristine, irinotecan, etoposide and paclitaxel are foremost examples of drugs stemming from a natural origin [1–3]. Development of strategies to improve the bioavailability and the stability have prior importance in the path of the natural compounds into clinic [4]. Low bioavailability largely arises from a diminished absorption level, instability in physiological conditions and incapability to bypass the lipid membranes. The net result of these drawbacks becomes loss or limitation of efficacy. Flavonoids, tannins, and terpenoids fall into this category [4, 5]. Among alternative approaches, nanoparticular drug delivery systems come forward to overcome these limitations and to benefit more from these natural compounds in medicine.

The isothiocyanates, and in particular, sulforaphane, have emerged to the spotlight with diverse biological activities including antioxidant, anti-inflammatory and anti-cancerous properties [6–9]. Sulforaphane displayed promising anti-cancer efficacy against colon cancer, pancreatic cancer and leukemia, yet its efficacy was particularly strong against breast and prostate cancer [10, 11]. Beside its biological effectiveness, sulforaphane is characterized with minimal toxicity. However, despite the mentioned benefits

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and high biosafety, certain molecular features of sulforaphane including (i) high sensitivity to oxygen, heat, and alkaline conditions, (ii) poor oral bioavailability, and (iii) water instability limit its usage in medicine [12]. Sulforaphane is only moderately stable over time, especially in aqueous solution [Sulforaphane Bioavailability from Glucoraphanin-Rich Broccoli: Control by Active Endogenous Myrosinase, Jed W. Fahey, W. David Holtzclaw, Scott L. Wehage, Kristina L. Wade, Katherine K. Stephenson, Paul Talalay]. Researchers propose nanomedicine as a suitable approach to direct this isothiocyanate compound toward the tumor cells. Indeed, various nanoparticular drug delivery systems including gold-coated iron oxide (Fe_3O_4) nanoparticles [13], sulforaphane-conjugated selenium nanoparticles [14], tellurium flower-like nanoparticles [15], polymer nanoparticles [16–18], iron oxide-gold core shell nanoparticles [19], liposomes [20, 21] and lipid-based nanoparticles [22, 23] were studied for their efficacy to deliver sulforaphane to breast cancer cells. As an alternative approach, this study introduces sulforaphane-loaded emulsomes to overcome stability problem of the compound and to enhance its efficacy.

Emulsomes represent lipid-based drug delivery systems with broad variety of therapeutic applications principally most suitable for compounds that have low solubility and/or stability in water. Emulsomes are tiny lipid assemblies with solid fat cores that have high load capacity in particular for insoluble active compounds [24–26]. In the lack of any surfactants, emulsomes offer high biocompatibility and safety profile [27]. Solid inner matrix of emulsome allow encapsulation of hydrophobic compounds and maintain a prolonged release profile [25, 26, 28, 29].

The present study introduces sulforaphane-loaded emulsomes as a novel drug delivery system and explores the potential of this new formulation to successfully deliver the sulforaphane to MCF7 breast cells *in vitro*. Characterization studies will be followed by cell culture studies including cell viability and IC_{50} analysis to investigate the anti-cancer efficacy and safety of the introduced nanomedicine approach.

Materials and methods

Materials

R-sulforaphane (High purity, 50 mg), glyceryl tripalmitate (tripalmitin, purity ≥99 %), 1,2-dipalmitoyl rac-glycero-3-phosphatidylcholine (DPPC, 99 %), and cholesterol (95 %), ethanol (99.8 %), chloroform (≥99.8 %), were purchased from Sigma-Aldrich GmbH, Germany.

Production of sulforaphane-loaded emulsomes

Tripalmitin, DPPC, and cholesterol were dissolved with a weight ratio of 20:2:1 (equivalent to 40 mg tripalmitin 4 mg DPPC, 2 mg cholesterol for 10 mL final emulsome production) in 1.5 mL chloroform. Sulforaphane (40 mg/mL), dissolved in 50 µL ethanol (equivalent to 2 mg sulforaphane), was added separately. The obtained mixture was placed in a rotary evaporator (BUCHI Labortechnik AG, Büchi, Switzerland) to remove the organic solvents under reduced pressure at 474 mbar and 54 °C. The formed dry film was hydrated with double distilled water, the temperature was set to 80 °C and the solution was rotated until the lipid film was resuspended at atmospheric pressure. After 4 h, the flask containing the sample was transferred into the ultrasonicator (Bandelin electronic, Berlin, Germany). The particles were homogenized in ultrasonication bath at 70 °C for 1 h. The obtained emulsome suspension was placed in ice for 10 min; then centrifuged at 11,000 rpm for 5 min to spin down unincorporated sulforaphane. The sulforaphane-loaded emulsomes in the supernatant were taken and stored at 4 °C until further analysis. Empty emulsomes were prepared with the same procedure but without addition of sulforaphane.

Characterization techniques

Physicochemical properties: size, polydispersity (PDI) and zeta-potential

Sulforaphane-loaded emulsome were diluted in 1 mM KCl solution (pH 6.3) and examined for their particle size distribution (Dynamic Light Scattering; DLS) and zeta potential characteristics as previously described by Bolat et al. [26].

Size, shape and morphology

Size, shape and morphology of emulsome formulations were analyzed using a scanning electron microscope (Zeiss EVO-HD-15). A short-term fixation pre-treatment method was used before the imaging process. In a nutshell, emulsome samples were put onto an aluminum holder and allowed to dry at 4 °C. The dried samples were fixated for 15 min using PBS containing 2.5 % glutaraldehyde. Following the removal of PBS containing 2.5 % glutaraldehyde, samples were washed three times in total over the course of 10 min with distilled water. The samples were subsequently subjected to gold-sputtering (EM ACE200, Leica), followed by SEM analysis.

Dispersity in water

To further examine how sulforaphane-loaded emulsomes behave as dispersed in an aqueous environment, confocal laser scanning microscopy (CLSM) study was carried out in addition to SEM analysis. A sample of 5 μL of emulsomes was placed on a glass. CLSM analysis was performed using the CLSM instrument LSM780 (Zeiss, Turkey).

Quantification of sulforaphane

High-performance liquid chromatography (HPLC) technique described by Han and Row [30] was used with slight modifications to quantify the amount of sulforaphane encapsulated inside emulsomes [30]. Accordingly, first to eliminate water from the system emulsomes were dried in a vacuum evaporator for 2 h. The concentrated emulsion was then mixed in ethanol at a ratio of 1:10. Centrifugation was applied at 18,800 g for 40 min. The supernatant was centrifuged, vortexed, and then put right into the collector of HPLC. During operation, 10 μL of the sample was automatically injected into the injection port and analyzed in Waters Symmetry C18 (75 mm × 4.6 mm × 5 μm) column (Waters Ltd., UK) at 35 °C using a 20:80 (v/v) acetonitrile:water ratio. Ultraviolet detector system Waters 2695 Alliance 2998 PDA (Waters Ltd., UK) was used at 220 nm to quantity the amount of sulforaphane in the samples. The peak area that was correlated with the standard curve was used to establish the compositional distribution of sulforaphane in the sample. Sulforaphane was eluted at retention time of 7.6 min during the 20-min total HPLC analysis time per sample.

Cell culture studies

Cell lines

Eagle's Minimum Essential Medium (EMEM) was purchased from Sigma-Aldrich GmbH (Germany) and Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12) media was supplied by Thermo Fisher Scientific (USA). Phosphate-buffered saline (PBS; Multicell, Wisent), fetal bovine serum advanced, heat inactivated (FBS; Capricorn Scientific), penicillin, streptomycin and amphotericin (Gibco) were purchased from Thermo Fisher Scientific (USA). MTS reagent (3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) (CellTiter96 AqueousOne Solution) was supplied by Promega, United Kingdom.

MCF7 cells were grown in a medium made up of EMEM with 2 mM glutamine, 1 % non-essential amino acids, 10 % FBS/FCS, and 1 % antibiotic/antimycotic (both PAA). MCF10A cells were grown in DMEM/F12 without L-lysine and L-arginine. To make the complete growth medium, the medium was supplemented with 5 % horse serum, 20 ng/mL epidermal growth factor, 0.5 g/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 ng/mL insulin. All cells were cultured at 37 °C in a humidified atmosphere (5 % CO2) condition.

Cell viability assay

Following the treatment of MCF7 and MCF10 cells with free sulforaphane (i.e., sulforaphane dissolved in ethanol), (empty) emulsomes and sulforaphane-loaded emulsomes, cell viabilities were evaluated using MTS assay. The manufacturer's instructions were followed when conducting the cell viability assay. In 96-well plates, 1×10^6 cells were seeded and then incubated for 24 h. The next day, 0, 5, 10, 20, 30, and 40 μM concentrations of free sulforaphane and sulforaphane-loaded emulsome were applied. To verify the safety of the lipid formulation, cells in the control group were treated with empty emulsomes with the exact same volume in the sulforaphane-loaded emulsome treatment groups. The growth medium was withdrawn after each incubation time (24, 48 and 72 h), and then 100 μL of the MTT assay was added to the serum-free culture medium. This mixture was then incubated for 4 h at 37 °C. The absorbance intensities at 570 nm were measured using the microplate reader. Triplicates of each sample were run on each experiment.

Statistical analysis

GraphPad Prism Software (version 6.01) was used to perform statistical analysis. Error bars represent standard error of the mean and the data sets were compared using two-way ANOVA and Student's t-test. Differences were considered statistically at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$.

Results

The particle size distribution (DLS) and zeta potential characteristics (Phase Analysis Light Scattering; M3-PALS) of formulations were determined for four distinct sulforaphane-loaded emulsome formulations. The average particle size and average zeta potential of the formulation were determined as 246.0±14.1 nm and −23.5±2.4 mV, respectively, where the plus-minus signs indicate the margin of average sizes of each production made of the same

composition. An average polydispersity index of 0.38 indicated a moderately polydisperse suspension, as the value lies between 0.1 and 0.4 [31]. Dispersed characteristics of the emulsome suspension was also visualized by confocal laser scanning microscopy analysis, where the small size of the particles beyond the resolution power of confocal did not allow detailed information. Yet, presence of any agglomeration was not observed (data not shown). Shape, size and surface morphology of sulforaphane-loaded emulsomes were inspected by SEM analysis. Spherical shape and smooth particle surface were seen as characteristics of these nanoparticles (Figure 1). Particle diameters were once again examined by drawing a line parallel to selected particles on the electron micrograph. The analysis supported the average particle size data provided by DLS data. Concentration of sulforaphane inside the formulation was estimated using highly sensitive HPLC technique. Retention time of sulforaphane was recorded as 7.6 min in both free sulforaphane and sulforaphane-loaded emulsome formulations (Figure 2). Based on these data, the corresponding concentration of sulforaphane inside emulsomes were calculated as 0.036 mg/mL.

Cell culture studies were performed on both MCF7 human breast cancer and MCF10A human breast epithelial cell lines. Sulforaphane concentrations in range of 1–100 µM were examined. Cell viability data displayed dose and time dependent manner decrease in viability of MCF7 cells for free sulforaphane and sulforaphane-loaded emulsome treatments (Figure 3A–C).

In the first 24 h, (free) sulforaphane was foremost active against MCF7 cells with a sharp decrease in viability to 32.7 % at 1 µM, 10.0 % at 5 µM and 3.2 % at 10 µM. Sulforaphane-loaded emulsomes were less active, where viability of MCF7 cells became 55.2 % at 5 µM and 33.9 % at 100 µM within the same period of time.

At 48 h, a slight recovery was observed in MCF7 cells treated with 1 and 10 µM (free) sulforaphane, whereas the percentage of viable MCF7 cells continued to decline in 5 µM sulforaphane treatment group: 51.1 % at 1 µM, 5.33 % at 5 µM and 9.5 % at 10 µM. At the same time, the viability of MCF7 cells treated with sulforaphane-emulsome decreased to 51.4 % at 5 µM and 27.4 % at 100 µM. Blank emulsomes decreased the cell viability of MCF7 cells down to 50.5 % at highest concentration.

At 72 h, the viabilities for MCF7 cells became 57.1 % at 1 µM, 4.6 % at 5 µM and 1.7 % at 10 µM (free) sulforaphane. Percentage of viability for MCF7 cells treated with sulforaphane-emulsome became 55.4 % at 5 µM and 23.1 %

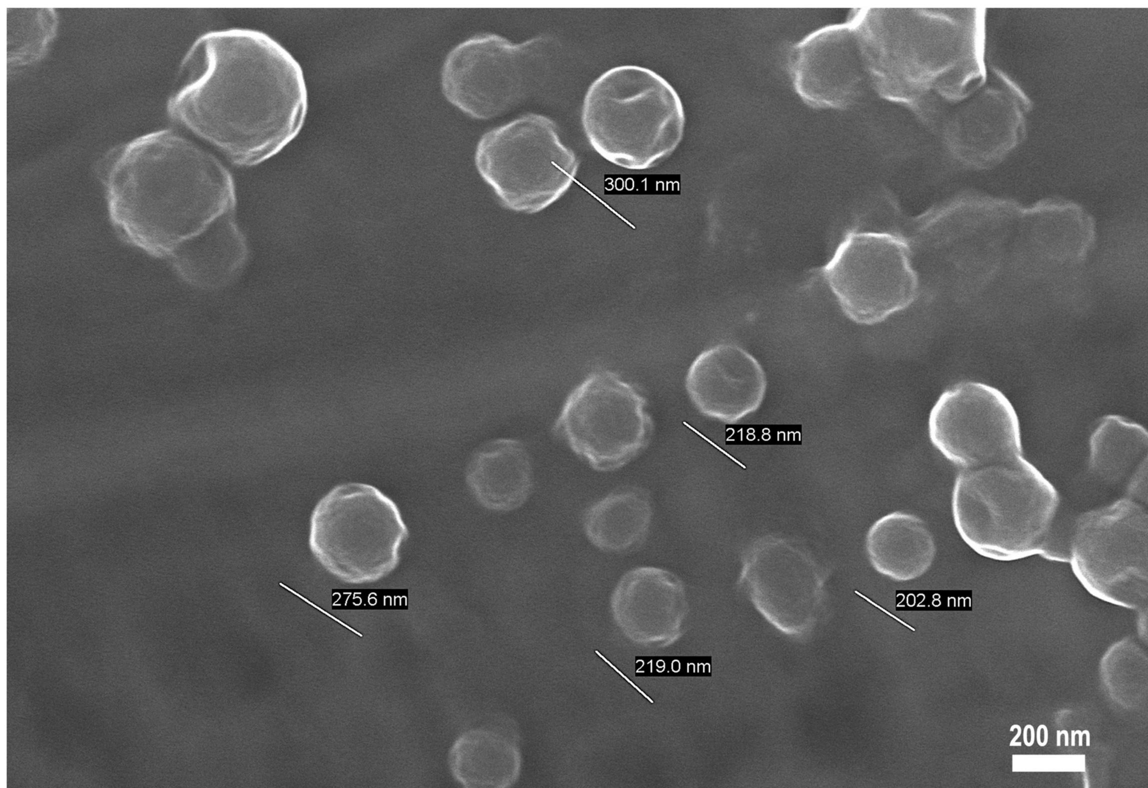

Figure 1: SEM image of sulforaphane-loaded emulsomes.

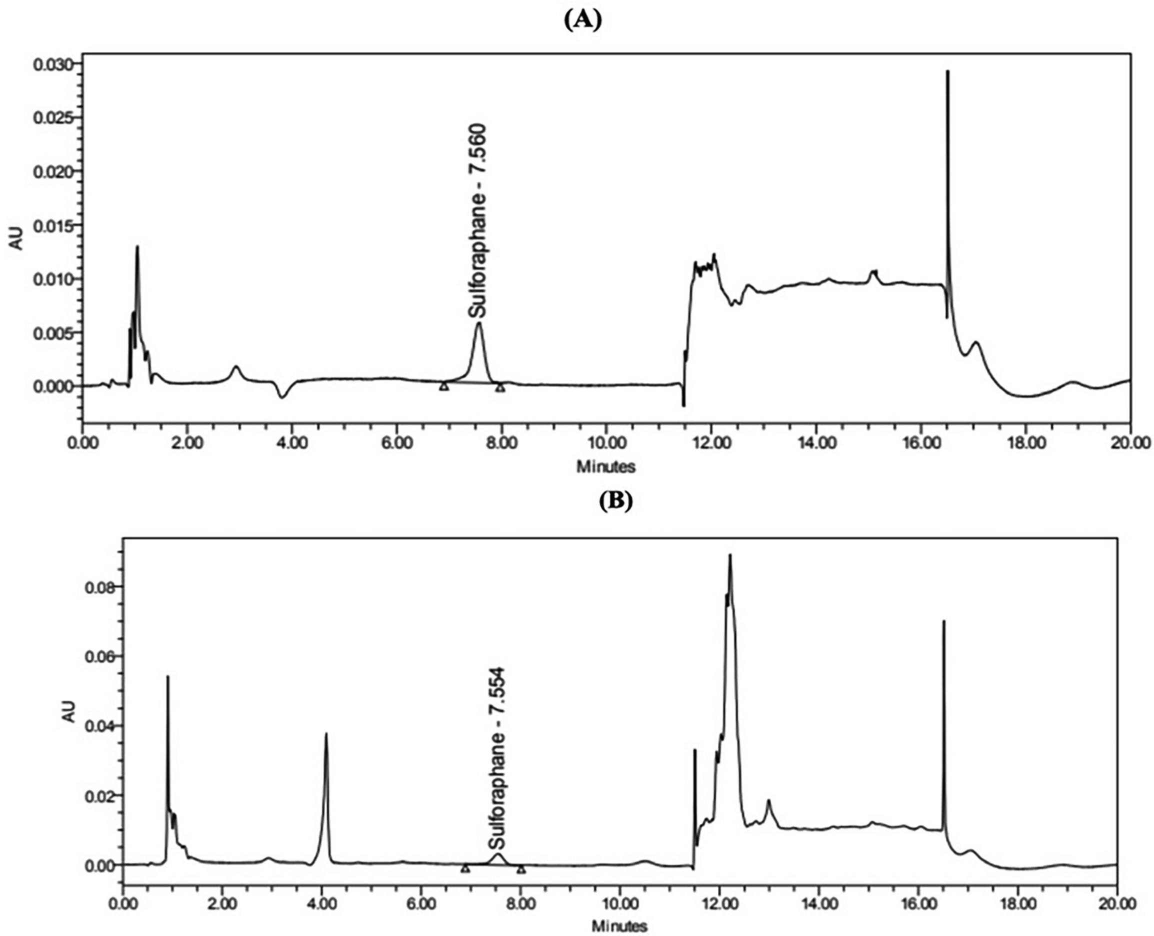

Figure 2: Chromatogram of time vs. absorbance intensity for HPLC analysis of (A) sulforaphane and (B) sulforaphane-loaded emulsomes.

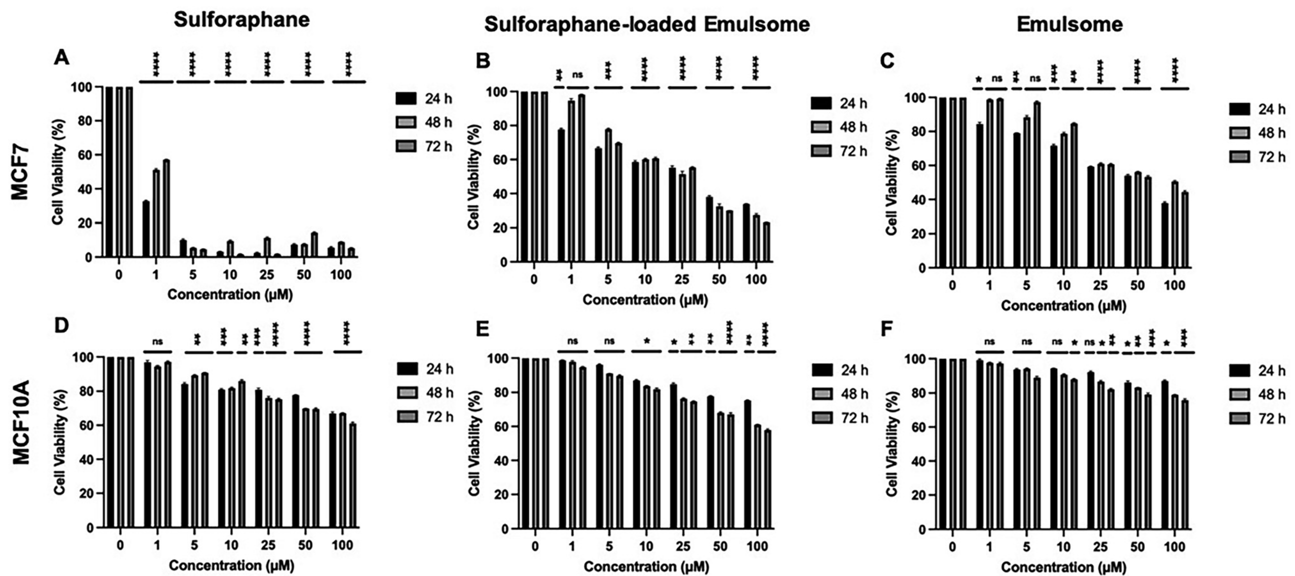

Figure 3: MTS cell viability analysis of (A–C) MCF7 human breast cancer and (D–F) MCF10A human breast epithelial cells treated with sulforaphane, sulforaphane-loaded emulsomes and blank (empty) emulsomes, respectively, for 24, 48 and 72 h. Statistical analysis for significance were done compared to negative control. Data represents mean (n=3) ± SD (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

at 100 µM. Blank emulsomes decreased the cell viability of MCF7 cells further down to 44.3 % at highest concentration, while blank emulsome concentrations equivalent to sulforaphane-emulsome concentrations of 10 µM and below seemed to be safe with ≥85 % cell viability.

Based on the cell viability data, IC_{50} value was calculated for both free sulforaphane and sulforaphane-loaded emulsomes using Graphpad Prism 9 Software. Based on the graph attained for average log concentration vs. cell viability, the IC_{50} values of free sulforaphane against MCF7 cells were deducted to be an average of 0.4 µM for 24 h, 0.9 µM for 48 h and 1.2 µM for 72 h. The IC_{50} values of sulforaphane-loaded emulsome against MCF7 cells were estimated as 23.4 µM for 24 h, 22.9 µM for 48 h and 21.11 µM for 72 h.

Treatment of MCF10A human breast epithelial cell lines with sulforaphane, sulforaphane-loaded emulsomes and blank emulsomes reduced viability of the cells in dose and time dependent manner, however the effect remained limited for all treatment groups (Figure 3D–F). Accordingly, free sulforaphane slightly decreased the cell viability to 96.8 % at 1 µM, 84.2 % at 5 µM and 80.9 % at 10 µM within 24 h 100 µM free sulforaphane could decrease the viability of MCF10A cells down to 66.9 %. Sulforaphane-loaded emulsomes were even less active against MCF10A healthy cells, where viability of MCF10A cells became 96.1 % at 5 µM and 75.3 % at 100 µM within the same time. At the same time point, viability of MCF10A cells remained always above 87 % for all concentrations of blank emulsomes.

At 48 h, percentage of viability became as the following for the MCF10A cells treated with (free) sulforaphane: 94.5 % at 1 µM, 89.4 % at 5 µM and 81.8 % at 10 µM. Percentages of cell viability for sulforaphane-emulsome treatment group declined to 91.1 % at 5 µM and 61.1 % at 100 µM. At 48 h, the viability of MCF7 cells remained above 79.0 % at highest concentrations of blank emulsomes.

The viability of MCF10A cells treated with free sulforaphane became 97.1 % for 1 µM, 90.9 % for 5 µM and 86.1 % for 10 µM sulforaphane at 72 h. Viability of MCF10A cells treated with sulforaphane-loaded emulsomes became 89.8 % at 5 µM and 57.9 % at 100 µM. For both free and emulsomal formulations, sulforaphane concentrations less or equal to 10 µM were observed to be harmless to MCF10A healthy cells, providing ≥80 % cell viability. Blank emulsome concentrations equivalent to sulforaphane-emulsome concentrations of 50 µM and below was observed to be safe for MCF10A cells (>80 % cell viability), while further increase in emulsome concentration decreased the cell viability of MCF10A cells down to 75.8 %.

Both sulforaphane and sulforaphane-loaded emulsome formulations did not yield any CC50 value, as the viability of MCF10A cells did not decline down below 50 % for any concentration.

Discussion

Since the relative instability of sulforaphane limits the medicinal applications of this effective compound, drug delivery systems come forward as a prominent approach to protect sulforaphane from degradation and deliver it to the targeted cell. In line with this perspective, the present study introduces sulforaphane-loaded emulsomes to improve bioavailability of the compound. The results of this study indicate that sulforaphane-loaded emulsomes can successfully entrap sulforaphane inside the inner solid matrix composed of tripalmitin surrounded by phospholipids up to concentrations of 0.036 mg/mL. As SEM analysis have suggested, spherical shape and smooth particle surface were characteristics of the emulsomes. The average particle size of the formulation was determined as 246.0±14.1 nm. While the DLS data with a polydispersity index of 0.38 was claiming the presence of a moderately dispersed suspension, SEM analysis indicated a narrower particle size distribution. As the reason behind, it can be speculated that sulforaphane-loaded emulsomes coming close to each other due to their lipid characteristics occasionally read out by DLS measurement as one larger particle. Yet, it is also worth to emphasize that no agglomeration was monitored in confocal laser scanning microscopy (data not shown). The negative average zeta potential value, i.e., −23.5±2.4 mV, seem to contribute the dispersity and stability of the particles in suspension.

Biological efficacy of sulforaphane-loaded emulsomes was studied *in vitro* on both MCF7 human breast cancer and MCF10A human breast epithelial cell lines. Free sulforaphane was found to be more effective than the sulforaphane-loaded emulsomes, which is not unexpected, because the previous studies on emulsomes already demonstrated that the uptake inside the cell and thereafter the release of poorly water-soluble compounds into the cytoplasm delay emulsome formulation to show their effect, thereby prolonging their biological activity [26, 28]. Delivered as dissolved in ethanol, free sulforaphane molecules could decrease the viability of MCF7 cells sharp to 32.7 % at 1 µM at 24 h 5 µM free sulforaphane was yielding cell viabilities close 5 % in only 48 h. Overall, IC_{50} values of 0.4 µM for 24 h, 0.9 µM for 48 h and 1.2 µM for 72 h explicitly show the potency of free sulforaphane against MCF7 cells. This data does not completely fit to IC_{50} values of sulforaphane in the literature, where a diversity in values is present. Li et al. [32] reported that IC_{50} of approximately 0.5–1.0 µM sulforaphane was capable of suppressing mammosphere formation on MCF7 sphere model [32]. In various studies, IC_{50} values such as 2.014 µM [33], 19 µM [34], 9.2 µM [35], 33.8 µM [36], 7.5 µM [37], 7.12 µM [38] and 31.2 µM [17] were reported for anti-proliferative effect of sulforaphane against MCF7 cells.

As prementioned, incorporated to emulsomes the efficacy of emulsomes seems to decrease. The IC_{50} values of sulforaphane-loaded emulsome against MCF7 cells were estimated as 23.4 µM for 24 h, 22.9 µM for 48 h and 21.11 µM for 72 h. Yet, the data of sulforaphane-loaded emulsomes were found to be comparable with the values in the literature. The IC_{50} value of the mPEG-PCL micelles against MCF-7 cells was found to be 14.21 µM [17]. Similarly, sulforaphane as loaded in PCL-PEG-PCL micelles was shown to have an IC_{50} value of 19.15 µM for 72 h [39].

In earlier studies, the uptake mechanism of emulsomes were shown by TEM analysis of thin-sectioned cells to occur via endocytosis [24]. Upon cell uptake, a sustained release of lipophilic load from the solid lipid core of emulsomes was observed by the help of confocal microscopy analysis, thereby also prolonging the biological effect of the incorporated compound [26, 28, 29]. Due to their larger size than the free molecule, emulsomes were monitored to enter into the cell with a certain delay compared to free molecules, where the level of delay must depend on the cell properties as well as the surface characteristics of the prepared formulation [25, 26, 28, 29, 40]. However still, the IC_{50} data of the current study contradicts with these earlier findings in terms of efficacy. While the exact reason behind remain uncertain, it might be speculated that the cell uptake mechanism of sulforaphane-loaded emulsomes with their negative zeta potential (i.e., 23.5±2.4 mV) does not occur fast, in particular not fast compared to free sulforaphane, and/or upon the uptake due to the prolonged release from the inner solid core to the cytoplasm sulforaphane does not reach to the eventually tested concentrations inside the cytoplasm, and a loss in efficacy ultimately occurs compared to free sulforaphane.

Cell culture study on MCF10A cells disclosed that both free sulforaphane as well as sulforaphane-loaded emulsomes are safe to use at concentrations equal or less than 10 µM. Further increase in the concentration results in decline of cell viability below 80 %, where the decrease seems not to be caused by the nanocarrier but the sulforaphane, because blank emulsomes display stable safe profile also at concentrations equivalent to 50 µM sulforaphane-loaded emulsomes. This final data highlights the biological safety of emulsome against MCF10A.

Conclusions

Sulforaphane is an isothiocyanate present within broccoli, well-known for its distinctive anti-cancer properties particularly against breast cancer. However, its low bioavailability necessitates researchers to seek applications of novel nanomedicine strategies. In this study sulforaphane-loaded emulsomes were developed and introduced for the first time as an alternative approach for the delivery of sulforaphane. Characterization part of the study described the stable, moderately dispersed features of the formulation, while cell culture studies assessed the anticancer efficacy of sulforaphane-loaded emulsomes on MCF breast cancer cells *in vitro* and particularly underlined the safety of the sulforaphane-loaded emulsomes.

In brief, as a prominent approach the present study highlights the potential of sulforaphane-loaded emulsomes to improve bioavailability of sulforaphane and deliver it to the targeted cell without interfering with its safety profile.

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Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. RK produced sulforaphane-loaded emulsomes, performed the characterization experiments, conducted cell viability analysis, and calculated IC_{50} values for sulforaphane and sulforaphane-loaded emulsomes. MHU designed the complete study, guided the conduct of experiments, and supervised data analysis. RK and MHU were actively involved in writing. MHU revised the manuscript. Both authors have read and approved the final manuscript.
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