

Microalgae-derived vesicles as potential carriers for therapeutic biomacromolecules

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ABSTRACT

Microalgae-derived vesicles (AGVs) were investigated as potential carriers for therapeutic biomacromolecules. AGVs were prepared by rehydration of a lyophilisate of proteolipid ghost membranes from *Dunaliella tertiolecta* and subsequent extrusion or sonication. Based on our results, extrusion is suggested to be a more suitable method because of the low loss of the lipid and protein content. Applying small-angle neutron scattering and dynamic and electrophoretic light scattering, we found that the formed AGVs are spherical, unilamellar, with moderate polydispersity, and a negative surface charge of -27 mV. EPR findings show that they retain the membrane dynamics, and possibly the degree of lipid chain saturation, of the 'mother' algal cells. The drug-binding efficiency of the reconstructed AGVs was tested for two model biomacromolecules, DNA and BSA, using an ethidium bromide exclusion assay and tryptophan autofluorescence, respectively. The electrostatic interaction between AGVs and DNA polyanion was mediated by Ca^{2+} ions, while for the interaction of BSA with AGVs hydrophobic interactions are assumed to play a dominant role. For both macromolecules tested, 40 % binding of the total drug amount was achieved by passive incubation, which indicates that reconstructed AGVs might expand the repertoire of potential delivery systems for therapeutic biomacromolecules.

1. Introduction

Therapeutic biomacromolecules include compounds over 1500 Da, like peptides, proteins, and nucleic acids, used in treatment of diseases, such as cancer or AIDS [1–3]. Delivery of these macromolecules in an active form is challenging due to their size, complexity and physico-chemical properties. Different carriers are involved to increase the therapeutic index by providing a protection of a drug from its microenvironment and improving stability, uptake, metabolism, and pharmacokinetic parameters [3,4]. The choice of carrier type is related to the desired therapeutic outcome.

Virus-based carriers are the most used in gene therapy for delivery of nucleic acids, thanks to their specificity and long effect [5]. However, they face limitations like immunogenicity, toxicity, production costs, and capacity [6,7]. Among non-viral carriers, lipid-based delivery systems (lipoplexes) are the most studied ones [8]. Lipoplexes are intended

to mask the negative charge of phosphate DNA residues to prevent electrostatic repulsion with the negatively charged surface of the cell membrane. The volume of the DNA molecule is reduced by condensation, which prevents its degradation by nucleases inside the cell. Cationic lipoplexes [9–11] target specific locations [7] but have cytotoxic and immunogenic limitations [12]. Negatively charged lipids (phosphatidylglycerol, PG, phosphatidylserine, PS, phosphatidic acid, PA), which benefit from lower clearance and phagocytosis by macrophages [12], were also tested for gene delivery. However, their negative charge prevents effective encapsulation of the DNA polyanion molecule due to electrostatic repulsion [13–15]. To overcome this problem, biogenic metal cations (Mn^{2+}) were suggested to mediate the interaction between DNA and the phospholipid bilayer [16]. Ca^{2+} showed the highest efficiency and lowest cytotoxicity in *in vitro* transfection tests on different cell lines [17]. It was attributed to high binding affinity of Ca^{2+} ions to DNA, resulting in effective condensation of DNA, as well as to

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Ca^{2+} -mediated promotion of lipoplex uptake by the cell [18].

Therapeutic peptides and proteins represent biological drugs with high specificity and potency, long-term effect, and reduced toxicity [19], used in various therapies and vaccines [20]. Despite their benefits, they face stability issues and can trigger immune responses [3,20]. Nano-delivery systems, including liposomes, can help to overcome these challenges by drug targeting, sustained release, and increased absorption [21–25]. The compositional attributes of the formulation, specially the physico-chemical properties of phospholipids [26] and the carried protein, such as polarity, charge, and molar mass [20], determine the way of protein presentation within the delivery system. Some liposomal protein formulations are already in clinical use, and many of them are in preclinical trials [21,24]. For fundamental drug delivery studies, employing different micro- and nanoparticles [27–29], fibrous scaffolds [28,30] or spray-dried nanosuspensions [31,32], bovine serum albumin (BSA) is used as a model protein, utilizing its structural similarity to human homologue (HSA). The ionisable residues give BSA the total effective charge, which depends on pH and contributes to its solubility [33]. Surprisingly, negatively charged BSA was found to adsorb on negatively charged mixed dipalmitoylphosphatidylglycerol:dipalmitoylphosphatidylcholine (DPPG:DPPC) liposomes, but did not adsorb on positively charged stearylamine:dipalmitoylphosphatidylcholine (SA:DPPC) mixed liposomes, indicating dominant hydrophobic interaction [34].

To sum up, liposomes represent a versatile and advanced nano-delivery systems for a wide range of biologically active compounds. The composition of clinically used liposomes includes lipids of natural origin (e.g. egg lecithin, cholesterol) or synthetic and semi-synthetic lipids [24]. Still, the biotechnology research seeks alternative bioinspired, sustainable and eco-friendly materials. Cell-based carriers mimic the complexity of natural proteolipid biomembranes and represent a new platform in drug delivery. These systems include: i) whole cell carriers; ii) cell membrane derived nanocarriers, known as ghost vesicles (GVs); iii) cell membrane clocked nanoparticles and iv) exosomes and microvesicle-based nanocarriers [35,36]. Among GV, bacteria-based [37–40] and erythrocyte-based GV [41–44] show promising results. The latest research indicates the possibility to exploit algae-based ghost vesicles (AGVs), derived from a unicellular marine microalga *Dunaliella tertiolecta*, as biological drug carrier systems [45]. Marine microalgae [46,47], microalgae-powered microrobots [48], microalgal extracts [49] and extracellular vesicles secreted by microalgae [50] have already been proposed for drug delivery in different biomedical applications. Advantageously, algae do not display significant cytotoxicity, nor induce a systemic immune response; on the contrary, they contain numerous beneficial bioproducts [51,52], what broadens the therapeutic potential of AGVs.

The final amount of carried drug is influenced by the preparation method, affecting liposome structure, size, lamellarity, and physico-chemical properties. These parameters determine liposome stability, internalization by cells, and the drug efflux rate [53]. We prepared proteoliposomes from a lyophilisate of *Dunaliella tertiolecta* ghosts. The motivation for keeping the chemical complexity of algae membranes was to retain the biocompatibility and, for further application, to potentially benefit from antioxidant, anti-inflammatory, and other health-promoting properties of algal bioproducts. The lyophilisation of ghost vesicles was introduced as a supplementary step in order to preserve the functional properties of ghost vesicles upon long-term storage. We tested two methods for preparing unilamellar vesicles from a suspension of rehydrated lyophilisate, extrusion and sonication. Using colorimetric assays, we determined losses in lipid and protein content during AGVs preparation processes. Electron paramagnetic resonance spectroscopy was employed to explore whether the fluidity and dynamics of *Dunaliella tertiolecta* cell membranes is preserved also in reconstructed AGVs. Dynamic and electrophoretic light scattering were used to evaluate the colloidal characteristics of AGVs and small-angle neutron scattering to confirm their spherical shape. The thickness of

the proteolipid membrane was assessed from the fitting of SANS curves by using a model of unilamellar vesicles. In the second part of the study, the ability of DNA condensation by reconstructed AGVs in the presence of Ca^{2+} ions was monitored by fluorimetry, using the ethidium bromide quenching method. Nephelometry was used to gain information on relative changes in the size of the supramolecular aggregates. The binding of BSA and its leakage from reconstructed AGVs was monitored using tryptophan autofluorescence. We found that both studied model drugs were bound to AGVs with an efficiency of 40 %. These results indicate that reconstructed AGVs might expand the repertoire of potential delivery systems for therapeutic biomacromolecules.

2. Materials and methods

2.1. Materials

The 5 mmol L⁻¹ NaCl stock hydration medium and 0.4 mol L⁻¹ CaCl₂ solution were prepared by dissolution of an appropriate amount of sodium chloride (Lachema, Czech Republic) and calcium chloride dihydrate (Sigma-Aldrich, USA), respectively, in ultrapure water (18.2 MΩ cm, LabAqua Bio; Latvia). Ferric chloride hexahydrate of reagent grade (Sigma Aldrich, USA) and ammonium thiocyanate ACS reagent (Sigma Aldrich, USA) were used for the preparation of the Stewart assay. Chloroform of spectral purity (Slavus, Slovak Republic), deuterium oxide (D₂O, deuteration degree min. 99.9 %; Sigma-Aldrich, USA), 1,2-dioleoylphosphatidylcholine (DOPC; Avanti polar lipids, USA) and the Quick Start™ Bradford Protein Assay kit (Bio-Rad, USA) were used as delivered. Calf thymus DNA (sodium salt), highly polymerized, was purchased from Sigma-Aldrich (USA). Ethidium bromide (EtBr; Merck, Germany) dissolved in NaCl stock medium was used as a fluorescent probe. Spin labels, 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) and 16-doxyl stearic acid (16-SASL) were purchased from Sigma (Hungary), 1-palmitoyl-2-stearoyl-(16-doxyl)-sn-glycero-3-phosphocholine (16-PCSL) from Avanti Polar Lipids (USA). Bovine Serum Albumin (BSA) for loading experiments was from Sigma (Hungary).

2.2. Sample preparation

AGVs were prepared by hypoosmotic shock of the photosynthetic marine microalga *Dunaliella tertiolecta* Butcher [54] as described in detail elsewhere [45,55]. The suspension of empty vesicles was stored overnight at -20 °C and then lyophilised for 24 h at -50 °C and 10 Pa (Freezone 2.5, Labconco, USA). Reconstructed AGVs were prepared as follows: the lyophilisate was hydrated with NaCl stock medium at a concentration of 1 mg mL⁻¹. D₂O was used as a solvent of hydration medium in small-angle neutron scattering (SANS) experiments at the concentration of AGVs 10 mg mL⁻¹. The dispersion was homogenised by repeated vigorous vortex mixing and mild intermittent bath sonication (K5LE, Kraintek, Czechia) and stored for 48 h in a refrigerator for equilibration. Unilamellar vesicles were prepared from the stock dispersion one day before the experiment by extrusion or by tip sonication. The extruded vesicles were prepared by 21 passes through 200 nm pores in a carbohydrate filter (Nuclepore, USA) using a Liposofast basic extruder (Avestin, Canada) fitted with two gastight syringes (Hamilton, USA) [56], at laboratory temperature. An odd number of passes were performed to avoid contamination of the sample by vesicles that might not have passed through the filter [57]. Sonicated vesicles were prepared using a Microtip ultrasonic membrane disintegrator (SFX 150, Branson, USA) equipped with a 1/8" titanium tip. 20 cycles at a total energy of 10,000 J, 50 % amplitude, and on/off = 500/60 s pulse regime were applied. The dispersion was poured into a glass tube and placed in the ice bath and in nitrogen atmosphere during sonication to prevent degradation. Titanium traces released from the tip during sonication were removed by swing-out centrifugation (Rotofix 32A, Hettich Zentrifugen, Germany) at 11,200×g (RCF) for 20 min. The supernatant was used for the measurements.

DNA solution was prepared by dissolution in NaCl stock medium at an approximate concentration of 1.7 mg mL^{-1} . The precise concentration of prepared DNA solution and its purity was determined using UV–Vis spectroscopy (Agilent 8453, Agilent Technologies, USA), according to equation $c_{\text{DNA}} = (A_{260} - A_{320}) \cdot (4.7 \times 10^{-5})$, where A_{260} and A_{320} are the absorbance values at wavelengths $\lambda = 260$ and 320 nm , respectively. The $(A_{260} - A_{320}) / (A_{280} - A_{320})$ ratio was 1.81, indicating that the DNA solution was sufficiently pure. Samples were prepared by mixing solutions of DNA and reconstructed AGVs to obtain AGVs:DNA = 5:1 mol/base ratio in each sample. After $\sim 10 \text{ min}$ CaCl_2 solution was added at an appropriate volume to obtain the required molar ratio of Ca^{2+} :DNA. The volume of samples was adjusted by NaCl stock medium. Samples were vortexed and after 20 min the solution of EtBr was added at a ratio of DNA:EtBr = 6:1 mol/mol. Samples were measured after 30 min incubation with occasional mixing at laboratory temperature.

For EPR measurements, 16-PCSL and 16-SASL were dissolved in ethanol (1.3 mmol L^{-1} and 2.6 mmol L^{-1} , respectively), while TEMPO was dissolved in ultrapure water (at 10 mmol L^{-1}) to prepare stock solutions. For the membrane dynamics study, the algal cell suspension and rehydrated AGVs were evenly distributed into Eppendorf tubes (1.70 mg of algal cells and 0.93 mg of AGV lyophilisate per tube for each sample, respectively). After centrifugation at $23,180 \times g$ (EBA 12 R, Hettich Zentrifugen, Germany) for 30 min at 4°C , the supernatant was discarded. The pellet was resuspended in $100 \mu\text{L}$ of water, incubated at 40°C for 10 min, shaken 3 times and then added to one of the two spin labels ($2 \mu\text{L}$ for 16-PCSL or $1 \mu\text{L}$ for 16-SASL) under vortexing. The concentration of 16-PCSL and 16-SASL in the sample was $0.026 \text{ mmol L}^{-1}$. Samples were further incubated at 40°C for 10 min and vortexed 3 times. TEMPO ($4 \mu\text{L}$ of 10 mmol L^{-1}) was added to the suspended, incubated pellets ($100 \mu\text{L}$) and incubated at 40°C for 10 min. After centrifugation at $23,180 \times g$ for 30 min at 4°C , $900 \mu\text{L}$ of the supernatant was removed and the remainder was suspended. Subsequently, each $100 \mu\text{L}$ of the labelled sample was transferred to a capillary tube and centrifuged at $1500 \times g$ for 20 min at room temperature (RT, $22\text{--}23^\circ \text{C}$), followed by removal of the supernatant.

In loading experiments, 4.88 mg of rehydrated and centrifuged AGVs were suspended in $250 \mu\text{L}$ of 0.04 mol L^{-1} BSA solution. After $3 \times 30 \text{ s}$ of sonication, followed by incubation for 10 min at RT and centrifugation for 5 min at $10,300 \times g$, the supernatant served for the determination of unloaded BSA, while the ghost pellet was used for leakage experiments: $250 \mu\text{L}$ of water was added to the pellet, followed by incubation for 10 min at RT and centrifugation for 5 min at $10,300 \times g$. The fluorescence of the supernatant was measured. The supernatant was then pipetted back to the pellet. The cycle of centrifugation, incubation, measurement of supernatant, and repipetting was repeated for the new datapoints.

2.3. Methods

2.3.1. Colorimetry

Losses in lipid and protein concentration during the extrusion or sonication process were determined by colorimetric assays according to Stewart [58] and Bradford [59], respectively. UV–Vis absorption spectra were acquired using a diode array spectrophotometer (Agilent 8453, Agilent Technologies, USA) at 25°C .

The Stewart assay utilises the ability of phospholipids to form a complex with ammonium ferrothiocyanate in chloroform solution. DOPC ($0.127 \text{ mmol L}^{-1}$) was used as a standard solution. The assay contained 2 mL of 0.1 mol L^{-1} dye reagent, different volumes of the lipid standard solution ($0\text{--}1 \text{ mL}$) and the required amount of chloroform, up to a final volume of 4 mL . Vials were vigorously vortexed for 30 s and spun for 10 min at $112 \times g$ (RCF) in a centrifuge (Rotofix 32A, Hettich Zentrifugen, Germany). The lower layer was analyzed; chloroform was used as a blank. A similar procedure was used for AGV sample solutions.

The Bradford assay involves the binding of the Coomassie Brilliant Blue G-250 dye to proteins. Bovine serum albumin (BSA) was used as a standard. To $50 \mu\text{L}$ of each standard ($0.125\text{--}1 \text{ mg mL}^{-1}$) and to AGV

sample solution 2.5 mL dye reagent was added. The vials were vortexed for 10 s and incubated at RT for at least 5 min. $50 \mu\text{L}$ of NaCl medium in 2.5 mL of dye reagent was used as a blank.

2.3.2. Dynamic and electrophoretic light scattering (DLS & ELS)

The morphological characteristics of reconstructed AGVs were assessed by light scattering using Litesizer 500 (Anton Paar, Austria) at $25 \pm 0.1^\circ \text{C}$. To determine the size and polydispersity of AGVs dynamic backscattering of incident light was collected over 20 runs with duration of a single run of 10 s. To determine the zeta-potential of AGVs, and of their complexes with DNA, ELS was monitored at a voltage of 200 V and a total number of 20 runs. Three independent measurements were performed for each sample. The Kalliope™ software supplied with the apparatus was used for analysis of the measured data. The hydrodynamic diameter was calculated using the Stokes-Einstein equation; to evaluate the zeta-potential the Smoluchowski approximation was used.

2.3.3. Small-angle neutron scattering (SANS)

In SANS, the scattered intensity, $I(q)$, is measured as a function of the momentum transfer, q , which depends on the wavelength, λ , of the incident neutron beam and the scattering angle 2θ ; $q = (4\pi/\lambda)\sin(\theta)$. The coherent scattering intensity for a system of monodisperse centrosymmetric particles is given by

$$I(q) = N \cdot P(q) \cdot S(q) \quad (1)$$

where N is the number density of particles, $P(q)$ is their form factor, and $S(q)$ is the interparticle structure factor describing the positional correlations among particles as a result of their mutual interactions. $S(q) \cong 1$ for dilute particles and those weakly interacting, such as ULVs prepared by extrusion at phospholipid concentration $< 2 \text{ wt\%}$ [60].

The experimental SANS curves were analyzed using the model of polydisperse unilamellar vesicles. The fitting was processed with SasView software, version 6.0.0 ([61], <http://www.sasview.org/>) using the built-in model of a vesicle. A unilamellar vesicle is represented by a hollow sphere with a shell of the thickness of the membrane (d_m). The geometry of the vesicle is shown in Fig. S2. The intensity of scattering is calculated [62] as

$$I(q) = \frac{\text{scale} \cdot \Delta\rho^2}{V_{\text{shell}}} \left[\frac{3V_{\text{tot}} j(qR_{\text{tot}})}{qR_{\text{tot}}} - \frac{3V_{\text{core}} j(qR_{\text{core}})}{qR_{\text{core}}} \right]^2 + b \cdot c \cdot g \quad (2)$$

$$j(x) = \frac{\sin(x) - x \cos(x)}{x^2}$$

where $j(x)$ is the spherical Bessel function, V_{shell} is the volume of the shell, scale is a scale factor, V_{tot} is total volume of vesicle with radius R_{tot} , V_{core} is the volume of the core with radius R_{core} ; $\Delta\rho = \rho_m - \rho_s$ is the contrast between the membrane and the solvent (D_2O), $b \cdot c \cdot g$ is background. The thickness d_m of the membrane is given by $d_m = R_{\text{tot}} - R_{\text{core}}$. The values of neutron scattering length density (NSLD) $\rho_m = 2.9 \times 10^{-6} \text{ \AA}^{-2}$ [63], and $\rho_s = 6.35 \times 10^{-6} \text{ \AA}^{-2}$ were used for the fitting. Polydispersity was included in fitting by convolution with a distribution of vesicles radius using Schulz function. The ideal model scattering curve is smeared by the instrumental resolution, $\Delta q/q \cong 20\%$. The presented errors include the uncertainty of the fit quantified by the reduced chi-square parameter.

Experiments were performed on YS-SANS diffractometer [64] at BNC (Budapest, Hungary). The temperature was maintained at 30°C and the scattering intensity was collected for 30 min. A setup covering a range of $q = 0.003\text{--}0.1 \text{ \AA}^{-1}$ at a neutron wavelength of $5 \text{ \AA}$ and $12 \text{ \AA}$ ($\Delta\lambda/\lambda = 20\%$ FWHM) and sample to detector distance of 5.3 m . The scattered neutrons were collected by a 2D position sensitive gas detector (LETI, Grenoble, France). Corrections for detector response, room background and sample transmission were carried out using BerSANS software.

2.3.4. Fluorescence spectrometry

The degree of condensation of DNA by algae-based ULVs in presence of Ca^{2+} ions was investigated utilizing EtBr quenching method [65]. The steady-state fluorescence intensity was measured using the Fluoromax-4 spectrofluorimeter (Horiba Jobin Yvon, France) at an excitation wavelength of $\lambda_{\text{ex}} = 510$ nm, i.e. at an isosbestic point for EtBr: DNA solutions [66]. Spectrofluorimetric cells with an optical path length of 1 cm (Hellma, Germany) were used and the emission intensity was recorded at $\lambda_{\text{em}} = 608$ and 683 nm for 120 s every 0.5 s at 8 nm slit widths. A temperature of 25 °C was controlled by a Peltier module (Wavelength Electronics, USA). Correction of fluorescence intensity for spectral response by monitoring the value of $S1c/R1c$, where $S1c$ and $R1c$ denote the signal detector and reference (lamp) detector corrections, respectively, for wavelength- and time-dependent response, was applied. Fluorescence intensity of the measured samples (I_S) was corrected for the intensity of the blank sample (EtBr in solvent, I_B) and then normalized to the intensity of the reference sample without CaCl_2 (I_R) using

$$I_{\text{norm}} = \frac{I_S - I_B}{I_R - I_B} \times 100 \quad (4)$$

where I_{norm} is the normalized intensity. The size of the aggregates was monitored nephelometrically by setting the monochromators at a wavelength of $\lambda_{\text{ex}} = \lambda_{\text{em}} = 600$ nm. The scattered radiation was detected at 90° and excitation and emission slit size of 1 and 1 nm, respectively. The experimental data were evaluated using FluorEssence v. 3.9 software working in the OriginPro® environment.

The experiments on loading/leakage of BSA were carried out at RT by a Fluorolog-3 (FL3-222) modular spectrofluorimeter (Horiba Jobin Yvon, Japan) at an excitation wavelength of 295 nm. Data analysis, fitting and visualization were performed using the *Igor Pro* program (Wave Metrics, Oregon, USA). Intensity correction was carried out by the subtraction of water as a blank sample.

2.3.5. Electron paramagnetic resonance spectroscopy

Continuous EPR measurements were performed by Bruker ELEXSYS-II E580 X-band spectrometer at RT and at 45 °C with the following instrument settings: scan range 3300–3400 G; microwave frequency 9.42 GHz; microwave power 9.464 mW; modulation frequency 100 kHz; modulation amplitude 0.5 G; field axis resolution 1024; scans 4; sweep time 40.96 s. Data fitting and evaluation were carried out with *Igor Pro* program (Wave Metrics, Oregon, USA).

2.3.6. Statistical evaluation

The ‘mother’ algal cell membranes used to prepare AGVs display substantial compositional variability arising from the standard intra-species biodiversity. This can lead to random error, considered as a significant component of the cumulative error in our experiments, increasing the imprecision of the data. To account for this uncertainty, all measurements were repeated 6-times, with three independent measurements being performed on samples prepared from two different batches of AGVs. The results are further subject to error of evaluation process, described in detail above. The data presented in the following tables and figures represent the average values obtained from repeated measurements, the corresponding errors indicate the standard deviation calculated by the error propagation method, taking into account both uncertainties.

3. Results and discussion

3.1. Membrane dynamics of reconstructed AGVs

The shelf life of liposomal formulations is related to their physical and chemical stability. Chemical stability means stability against hydrolytic and oxidative degradation of phospholipid structures. The level

of thermal/air autooxidation sensitivity is given by the amount of oxidisable lipids in the liposomal membrane. The hydrolysis type of chemical degradation of ester linkages in the phospholipid structure depends on pH and occurs most slowly at pH 6.5 [67]. Oxidised lipids can alter membrane properties such as fluidity, permeability, and phase behaviour [68]. To avoid this problem, storage of AGVs in a lyophilised state was suggested [69]. In addition, microalgae possess natural anti-oxidant activity [70]. This property was shown to be preserved in hydrolysates derived from specific microalgae by enzymatic hydrolysis [71]. We have investigated whether proteolipid vesicles prepared by rehydration of the AGVs lyophilisate with a water medium at a pH close to neutral values preserve the membrane fluidity.

We applied three types of spin labels (Fig. S1A–C) to compare the membrane dynamics of algal cells and AGVs. The membranes were studied in both the gel and fluid phase, at RT and at 45 °C, respectively. The three spin labels provide complementary information about the biophysical state of the membranes: i) 16-PCSL is a lipid type spin label. With the nitroxyl group in the position of C16 it gives information about the middle of the membrane. We found that a mobile membrane component is significant in both algal cells and AGVs, while a little immobile component was also noted (Fig. S4A). The immobile component originates from spin-labelled lipids tightly attached to the intramembranous surface of proteins [72–74], which could be an indication of a higher protein/lipid ratio in AGVs. The noisy spectra for AGVs at both temperatures also support some structural difference leading to less efficient incorporation of the spin label than into algal cells (not shown). The rotational correlation time (τ_C) of the 16-PCSL nitroxyl group displays a higher sensitivity to increasing temperature (from RT to 45 °C) in algal cells than in AGVs (Table 1). These differences may also be triggered in part by the smaller size of AGVs compared to whole cells. ii) The EPR spectrum of the 16-SASL stearic acid spin label (Fig. S4B), which has a single acyl chain, shows the presence of a mobile membrane component (different from the signal of 16-SASL in water). This spin label is incorporated at a slightly higher level into algal cells than into AGVs. Incorporation is more effective in the fluid phase, as expected. In the case of algal cells, τ_C decreases slightly with increasing temperature, as opposed to AGVs, where τ_C is temperature independent, and the values are between the τ_C values obtained for algal cells (Table 1). iii) TEMPO is a small water-soluble spin label. In a membrane-water dispersion it partitions between the membrane and the water phase (see, for example, [74]). Fig. 1 shows the normalized composite spectra (including both aqueous and membrane components) of the samples.

The decomposition of the spectra (Fig. 1A), according to Ref. [74], showed that the contribution of the membrane component is greater than 50 % for algal cells, and represents 25–40 % for AGVs. The rotational correlation time values of TEMPO for the algal cells were lower than for AGVs, but the increase in temperature decreased τ_C of both samples (Fig. 1B and Table 1, similar values are also reported for TEMPO in AGVs in our related publication [69]). Our results show that AGVs retain an overall rotational dynamics comparable to that of algal cells, suggesting lack of significant changes in lipid chain saturation or

Table 1

Rotational correlation times (τ_C) of membrane components for algal cells and reconstructed algal ghost vesicles (AGVs) at room temperature (RT) and at 45 °C obtained by respective spin label.

Spin label	Sample	τ_C (ns)	
		RT	45 °C
16-PCSL	algal cell	1.4 ± 0.1	1.1 ± 0.1
	AGVs	1.4 ± 0.2	1.4 ± 0.1
16-SASL	water	0.05	0.03
	algal cell	1.50 ± 0.01	1.2 ± 0.2
TEMPO	AGVs	1.3 ± 0.2	1.3 ± 0.2
	water	<0.003	<0.003
	algal cell	1.0 ± 0.1	0.73 ± 0.04
	AGVs	1.2 ± 0.3	1.1 ± 0.1

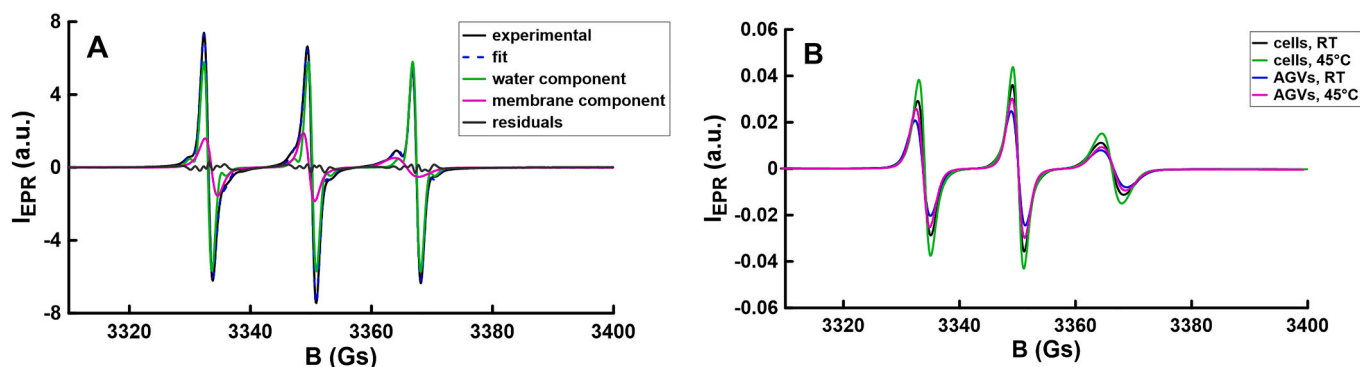


Fig. 1. (A) Example of decomposition of EPR spectra obtained for TEMPO spin probe. (B) Normalized composite EPR spectra of TEMPO in algal cell and reconstructed ghost vesicle (AGVs) membranes at room temperature (RT) and at 45 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peroxidation, hence preservation of their antioxidant activity. (A more direct evidence for the antioxidant activity of AGVs is presented in our related publication [69].)

3.2. Phospholipid and protein content of reconstructed AGVs

Lyophilisation of liposome systems without appropriate stabilizers leads to fusion of vesicles [67]. When hydrated, lipid molecules self-assemble into multilamellar vesicles (MLVs) composed of a number of concentric lipid bilayers separated by water layers. Protein components, depending on their physico-chemical properties, intercalate into the lipid bilayer or bind to its surface. Unilamellar vesicles (ULVs), composed of a single bilayer, are preferably used in carrier systems instead of MLVs, as they provide several times larger surface area available to interaction with transportable drugs. Moreover, in ULVs the influence of interlamellar interactions on size and stability of vesicles is eliminated [75]. We have prepared ULVs from rehydrated algal ghost lyophilisate by extrusion or sonication, two common methods used for the preparation of lipid vesicles [76]. Sonication generally provides ULVs of small size (typically 15–50 nm), however lipid and protein composition and concentration, as well as temperature and sonicator tuning influence the mean size and size distribution. Advantages of extrusion method include the applicability for relatively high lipid concentrations, absence of detergents, and productions of large ULVs with uniform size distribution. Extrusion was previously reported to be utilised as a convenient method in the preparation of vesicles from protein containing membranes of plant tissues [77,78] and mammalian cells [79].

Freshly prepared AGVs consist of three main components, proteins: lipids:carbohydrates, in an approximate ratio of 1:0.1:0.5 wt/wt/wt [69]. Proteins are mainly represented by globular surface proteins and by about 50 different types of proteins originating from the cytoplasm and chloroplasts [55]. In fatty acids and lipids, saturated and unsaturated acyl chains are almost equally distributed. Polar lipids represent ~80 % of total lipids in AGVs, with a contribution of PGs up to ~22 % [69]. During the preparation of the reconstructed AGVs by extrusion, we noticed traces of the sample trapped on the filter, whereas centrifugation following the sonication procedure resulted in a relatively large amount of sediment. This led us to evaluate the loss in lipid and protein content for each method. The protein concentration in the samples was determined using the Bradford assay. The calibration line was prepared for the BSA standard by measuring the absorbance at 595 nm. The lipid concentration was estimated using the Stewart assay by measuring the absorbance at 478 nm. The calibration line was constructed for the DOPC standard. We are aware that Stewart's assay is not recommended for samples where mixtures of unknown phospholipids may be present and especially its inability to quantify phosphatidylglycerols. Therefore, the loss of lipids was evaluated as a percentage of the lipid content prior

to extrusion or sonication. The percentage loss in phosphatidylcholines reflects the average percentage loss of all lipid content, since the losses were caused by mechanical trapping of lipid molecules on a filter during the extrusion and not due to their chemical binding conditioned by the structure of the lipid headgroup.

Table 2 summarizes the percentage of loss of the lipid and protein content in reconstructed AGVs prepared by extrusion or sonication. During the extrusion process, the loss of lipid content was found to be negligible, while it caused a loss of more than 10 % of proteins. A total of 1 mg of membrane proteins was previously suggested as the upper limit for extrusion [78]. This condition was fulfilled in our experiment. Thus, the observed loss of proteins might be due to the release of part of transmembrane proteins from membranes, as proposed in case of erythrocyte ghost preparation [80]. These proteins are believed to be surrounded by part of their original lipid environment and aggregated. Even more likely, this loss is related to the removal of membrane surface-bound globular proteins, as will be discussed later. Still, the value obtained after extrusion is three times lower compared to the loss of proteins using the method of sonication. Sonication also led to a more pronounced decrease in the lipid content, reaching ~17 %. This is presumably caused by the removal of a certain amount of lipids and proteins into the sediment during the process of centrifugation. Using the data from Table 2 we have calculated that the final composition, in terms of protein:lipid ratio (wt/wt), is 8.8:1 and 7.7:1 for extruded and sonicated AGVs, respectively. Thus, vesicles reconstructed by extrusion better maintain the composition, 10:1 wt/wt [69], of original ghost vesicles.

3.3. Morphology of reconstructed AGVs

The size and polydispersity of reconstructed AGVs prepared by extrusion or sonication were checked by DLS. A fast-exponential decreasing course of correlation function was obtained for all samples measured (Fig. 2A inset), what indicates a presence of quickly moving particles of small size [81]. The distribution of vesicle size was obtained by exponential fit of the experimental data as a function of the intensity weighted relative frequency on the hydrodynamic diameter of the vesicles (D_h). Broadness of the size distribution is given by the polydispersity index (PDI). A slightly asymmetric size distribution was typical for sonicated AGVs, while a relatively symmetric distribution for

Table 2

Percentage loss of lipid and protein content of vesicles prepared from dispersion of algal ghost lyophilisate by extrusion or sonication.

Vesicle preparation method	Loss of lipids (%)	Loss of proteins (%)
Extrusion	1.3 ± 0.3	13.2 ± 1.4
Sonication	17.3 ± 0.5	36.4 ± 1.8

extruded AGVs (Fig. 2A). The values of D_h and PDI obtained for the studied samples are summarised in Table 3. Extrusion provided vesicles of slightly larger size and polydispersity compared to sonication. However, given the standard deviation and the relatively high PDI value, the absolute difference in D_h is negligible and the vesicles obtained by the two methods can be considered to be comparable in size. To note, D_h obtained by DLS technique includes the size of the soft colloidal particle plus the thickness of the hydration shell, which can reach about 10–20 nm [82]. Moreover, the radius of the extruded vesicles is affected by the flow rate through the pores of the filter, which depends on the applied pressure, but is independent of the lipid properties [83]. This could explain the slightly larger size of the prepared vesicles than the size of the pores in the filter used. To the best of our knowledge, to date there is no experimental evidence about the effect of protein compounds on the extrusion process. On the other hand, lipid bilayer in sonicated vesicles dispose with high curvature energy, thus it is metastable. Additionally, the high portion of protein components determines the critical spontaneous radius of curvature of the vesicle bilayer. This energetic frustration is relaxed by fusion to vesicles of higher diameter, what might explain the unexpectedly high D_h and relatively high PDI values obtained after sonication. Surprisingly, the PDI value of extruded AGVs was comparable, within the experimental error, with PDI of sonicated AGVs (Table 3). This is likely caused by uneven distribution of components (predominantly proteins) among extruded AGVs. Generally, for a given sample concentration, the higher is the size of the ULVs formed, the lower is their number. This may lead to higher intervesicular variability of membrane components [78].

Small ULVs are more desirable in pharmaceutical applications than large vesicles. Large vesicles have decreased circulation stability and half-lives, and their unfavourable pharmacokinetic behaviour *in vivo* following injection makes them unsuitable for use in ligand-mediated and other forms of cell targeting [5]. On the other side, very small vesicles present a limitation in terms of the concentration of entrapped drugs. For drug delivery in gene therapy vesicles with diameters of 50–200 nm are currently used. The upper size limit is determined by the clathrin-mediated pathway of lipoplex endocytosis [84]. We propose that the size of reconstructed AGVs could be tailored by modifying their composition. Increase in the lipid:protein ratio is assumed to result in a reduction in vesicle size.

The size of the ULVs is affected also by their surface charge [85]. The method of ELS was used to determine the charge of vesicles by

Table 3

Hydrodynamic diameter (D_h), polydispersity index (PDI) and zeta-potential (ζ) of vesicles prepared from dispersion of algal ghost lyophilisate by extrusion or sonication. The thickness d_m of the proteolipid membrane and the inner radius R_{core} of the vesicle obtained from the analysis of SANS curves (asterisk denotes mean values and standard deviations obtained from 10 fittings of $I(q)$ vs. q for sonicated AGVs).

Vesicle preparation method	D_h (nm)	PDI (%)	ζ (mV)	d_m (nm)	R_{core} (nm)
Extrusion	215 ± 6	29 ± 5	−27 ± 2	15 ± 4	96 ± 7
Sonication	190 ± 4	34 ± 7	−28 ± 2	21 ± 3*	107 ± 28*

measuring their zeta-potential ζ . Most of the vesicles' surface charge is neutralized by the tightly bound counterions in the Stern layer. The remaining charge is balanced by the diffuse layer of the counterions extending to the bulk phase. The zeta-potential is defined as the electric potential of a particle at the shear plane, i.e. at a plane separating the two layers of counterions. For reconstructed AGVs prepared by both methods, the zeta-potential value was comparable within the experimental error, reaching nearly −30 mV (Table 3). The charge distribution was found to be symmetric and monotonously decreasing (not shown). This relatively high zeta-potential value is contributed by the high proportion of negatively charged PG lipids as well as by algal membrane proteins made up of different kinds of negatively charged amino acids [69]. The zeta potential of the particles is a key indicator of the stability of a colloidal dispersion. Absolute values of zeta-potential greater than 30 mV are considered indicative of stable dispersions, without a tendency to aggregate [81]. Our results show that the value of the surface charge is not much affected by the method of AGVs preparation, and that stable AGVs were present in all samples studied.

ULV dispersions prepared from synthetic neutral lipids by extrusion through 100 nm pore filters (or larger) are known to contain a high portion of multilamellar vesicles [86]. Addition of charged lipids or modification of neutral lipids by polyethyleneglycol was suggested to promote unilamellarity of extruded vesicles. In contrast, sonication was shown to produce essentially unilamellar vesicles even in the absence of charged lipids [75,87]. DLS size analysis relies on the assumption of non-interacting particles of spherical shape present in the investigated suspension. To confirm formation of spherical structures – proteolipid vesicles, thus to corroborate the relevance of results obtained by DLS,

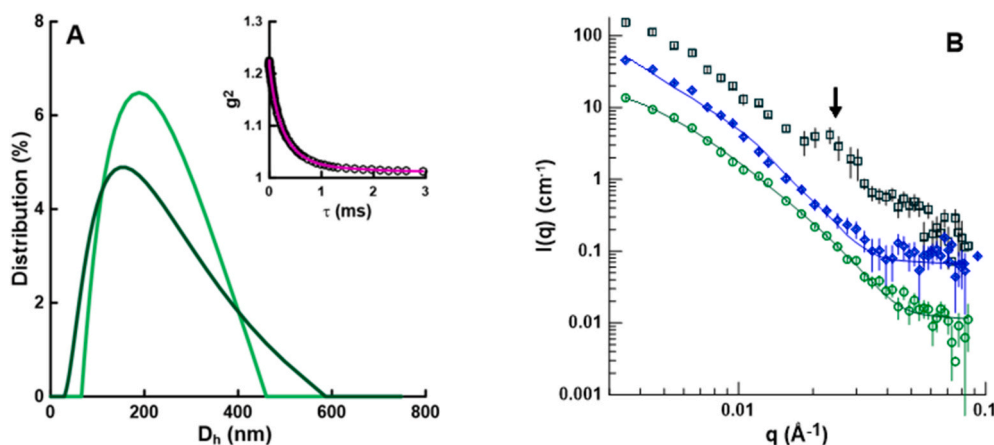


Fig. 2. (A) Intensity weighted size distribution of AGVs prepared by extrusion (light green) or sonication (dark green) of hydrated algal ghost lyophilisate as a function of hydrodynamic diameter D_h at 25 °C. (A inset) Temporal evolution of the pair-correlation function g^2 (black circles) and the best exponential fit of the experimental data points (purple curve). (B) SANS curves of vesicles prepared by hydration of algal ghost lyophilisate (black squares) and by consequent extrusion (green circles) or sonication (blue diamonds) of the dispersion. Full green line represents the best fit of $I(q)$ vs. q using a model of unilamellar vesicle ($\chi^2 = 1.22$) for extruded AGVs. Full blue line shows an example of congruity ($\chi^2 = 1.48$) as discussed in the text. For clarity, the intensity of untreated and sonicated AGVs was multiplied 20- and 6-times, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and to check the lamellarity of reconstructed AGVs, we performed a small angle neutron scattering experiment (SANS). This technique takes advantage of the contrast between the coherent neutron scattering length densities (NSLD) of AGVs membrane and solvent (D₂O). Fig. 2B shows SANS curves of dispersion of AGVs without treatment, AGVs after extrusion and sonication, respectively. To avoid the overlap of curves, the values of $I(q)$ for untreated AGVs were multiplied by a factor of 20, and $I(q)$ of those sonicated by a factor of 6. The scattered intensity $I(q)$ is proportional to the square of the contrast $\Delta\rho = \rho_m - \rho_s$ between the AGVs membrane (ρ_m) and solvent D₂O ($\rho_s = 6.36 \cdot 10^{-6} \text{ \AA}^{-2}$, at 25 °C). AGVs are rich in proteins. The mean NSLD for the hydrated protein in D₂O is $\rho \sim 2.9 \cdot 10^{-6} \text{ \AA}^{-2}$ [63], while for the proteolipid mixture rich in lipids we found $\rho \sim 0.37 \cdot 10^{-6} \text{ \AA}^{-2}$ [88]). It implies that the SANS intensity $I(q) \sim 10 \text{ cm}^{-1}$ observed at low q (Fig. 2B) is approximately 10 times lower compared to the scattering on vesicles of mixtures with the dominant fraction of phospholipids prepared in D₂O (see, for example, [88]).

The SANS curve of the untreated AGV dispersion shows a small peak at $q \sim 0.022 \text{ \AA}^{-1}$ (marked by an arrow on Fig. 2B), indicating the positional correlation between particles, thus the interparticle structure factor $S(q) \neq 1$ (equation (1)). Proteins are complex molecules, with nonuniform shapes as well as irregular domains of charged and hydrophobic surface amino acid residues. Because of the charges, proteins can interact electrostatically, and the nature of protein-protein interactions depends on the solution characteristics such as concentration, pH, ionic strength, temperature, etc. A direct consequence of this interaction is that the interparticle structure factor $S(q)$ becomes significantly depressed from unity in the small q -region (for example, see the pioneering study ([63], and references therein).

On the other hand, the scattering curve of AGVs prepared by extrusion does not show any peak (Fig. 2B, green circles). The experimental scattering curve was fitted by using a model of unilamellar vesicle. These structures are modelled by polydisperse hollow spheres with a layer separating the inside and outside aqueous compartments. (For further details, we refer readers to Ref. [89]). Fig. S2 shows the geometry of the vesicle with marked parameters of interest. The best fit of the experimental $I(q)$ is shown in Fig. 2B with full green line ($\chi^2 = 1.22$). The output of the fitting is the thickness of the proteolipid membrane, $d_m = 15 \pm 4 \text{ nm}$, and the inner radius of the vesicle $R_{core} = 96 \pm 7 \text{ nm}$ (Table 3). The value of the inner radius of vesicles is reasonable in respect to diameter of vesicles D_h determined by DLS (Table 3). In addition, the SANS curve of extruded AGVs was treated by a model-independent analysis based on Kratky – Porod plot [90]. The analysis is shown in the supplementary material (SANS analysis, Kratky – Porod plot, Fig. S3). The derived parameter, $d_g = 13.6 \pm 0.5 \text{ nm}$, related to the membrane thickness is in excellent accord with d_m obtained from the fitting. The thickness parameter d_g was previously shown to be a linear function of the transbilayer phosphate-phosphate distance d_{HH} in unilamellar phosphatidylcholine vesicles [91]. Moreover, the linearity of Kratky-Porod plot is a sensitive tool to uncover the unilamellarity of vesicles [88,92].

Fig. 2B, blue diamonds show the SANS on a dispersion of algae ghost vesicles prepared from hydrated lyophilisate and treated by tip sonication, as described in Materials and Methods. There is an obvious similarity in the two SANS patterns (extruded and sonicated AGVs), both show scattering on spherical objects. Full blue line represents a fit giving the values $d_m = 18 \pm 1 \text{ nm}$ and $R_{core} = 142 \pm 67 \text{ nm}$ ($\chi^2 = 1.48$). However, one can recognize non-perfect congruity between the fitting blue line and experimental points at detailed inspection of the pattern. In fact, we have obtained scattered values ranging for d_m between 17 and 25 nm and R_{core} between 83 and 157 nm with satisfactory quality of the fit ($\chi^2 \sim 1.15\text{--}1.57$). The mean values of d_m and R_{core} and standard deviation for sonicated AGVs are given in Table 3. The obvious difference between the two SANS patterns we attribute to the sample itself and the span between its preparation and measurement. While extruded AGVs were exposed to SANS not later than $\sim 6 \text{ h}$ after the preparation, the sample of sonicated AGVs was measured ~ 2 days after the treatment.

Generally, vesicles prepared by tip sonication are of smaller size that might result in their structural instability due to high curvature of the membrane, as we discuss in previous paragraph. Anyway, these results prove that the objects formed after reconstruction of AGVs by extrusion or sonication may well be defined as unilamellar spherical vesicles. This agreement is in line with the finding that the molecular and nanoscale organization of the bilayers does not depend on the manner of vesicle preparation method. Rather, the molecular scale organization of the bilayers is determined by interactions between the membrane components, which themselves do not depend on the method of the bilayer formation [93]. Thus, the slight differences in protein:lipid composition determined for the two methods (Table 2 and corresponding discussion) do not appear to affect the structure of the reconstructed AGVs.

The literature is poor in information on the thickness of the membrane of ghost vesicles prepared from *Dunaliella* cells. To our knowledge, the only information, obtained from the atomic force microscopy (AFM) profile of the deflated vesicles, suggests the thickness of the membrane $\sim 100 \text{ \AA}$ [45,94], and the thickness $\sim 90 \text{ \AA}$ of the envelope of the algae cell, estimated from the electron microscopy image [95]. It is evident that the thickness of the membrane formed by phospholipids (typically $\sim 5 \text{ nm}$) is much smaller compared to that obtained for the proteolipid membrane of AGVs in this study. Two interrelated points should be considered: the composition of the membrane, and the chosen approach. It is known that dissociable protons of proteins can be rapidly exchanged with deuterium in the D₂O solvent that leads to a decrease in the contrast $\Delta\rho$, and therefore structural parameters can be affected consecutively [96]. In pure D₂O, it is usually estimated that $\sim 80 \%$ of the labile protons are accessible to the solvent and hence exchange with deuterium atoms [63]. The large portion of proteins present in the algae-based system studied are globular surface proteins [55]. When the lyophilisate is hydrated, these proteins are supposed to attach to the surface and form a kind of corona around the self-assembled lipid vesicles; only a part of the proteins is reconstructed into bilayers. A similar arrangement was observed for reconstructed proteolipid MLVs derived from central nervous system myelin [97]. Thus, we speculate, that the derived d_m reflects the thickness of the membrane of self-assembled ghost vesicles composed predominantly of lipids, fatty acids, and integral proteins, i.e. components supporting the stability of the biological membrane of the algae cell. In this context, the peak in the SANS curve of untreated AGVs (Fig. 2B) detected at $q \sim 0.022 \text{ \AA}^{-1}$ indicates a correlation at a distance of $d \sim 28 \text{ nm}$, not observed after the treatment. The origin of the peak can be either in the interaction between charged proteins attached to the surface of the rehydrated AGVs that were released during the process of the treatment; or an appreciable amount of paucilamellar or multilamellar vesicles present in the mixture. The resulting repeat distance, $\sim 28 \text{ nm}$, is more than 2 to 4 times higher compared to reconstructed proteoliposomes of different origin [88,98,99]. The main difference of these systems is that they contain incomparably fewer proteins than algae ghosts.

3.4. Interaction of reconstructed AGVs with DNA

A threshold value for algae-ghost membrane permeability was determined to be 20–30 kDa [55], thus an effective interaction of macromolecules (DNA, $M_w = 8,580,000 \text{ g/mol}$ [100], what corresponds to 8,580 kDa, and BSA, $M_w = 66.5 \text{ kDa}$) with AGVs is expected. The interaction of reconstructed AGVs with DNA was followed by fluorescence spectrophotometry using ethidium bromide (EtBr, Fig. S1D) quenching assay [65,101]. This assay is frequently used to obtain qualitative information about the binding capacity of different lipid-based carriers for DNA [16]. EtBr is a cationic compound, which strongly binds to negatively charged DNA sites with one molecule for every 4 or 5 bp steps. The main binding modes include intercalation between the terminal base-pairs of the DNA, and electrostatic interaction with DNA phosphate backbone [102]. Four planar rings of EtBr molecule provide a fluorescence with a maximum at $\sim 605 \text{ nm}$.

Fluorescence increases up to 25-fold when binding to double-stranded DNA, due to isolation of the ethidium cation from the solvent. As a consequence, the proton transfer from an amino group to the solvent, causing a non-radiative decay in fluorescence intensity of non-bound EtBr, is eliminated [101].

Based on results presented above, AGVs prepared by extrusion were used for preparation of lipoplexes. The fluorescence spectra of the DNA + EtBr + AGVs samples showed two maxima (Fig. 3A). The first, at $\lambda_{\max 1} = 606$ nm, corresponds to the emission of DNA + EtBr complex, while the second, at $\lambda_{\max 2} = 683$ nm, fits to the maximum observed in spectrum of pure AGVs. We assume that this fluorescence is caused by residual chlorophyll in AGVs [45]. Since both, AGVs as well as DNA, are negatively charged, we used Ca^{2+} ions to mediate the formation of anionic lipoplexes. Generally, several electrostatically based binding events should be considered in this multicomponent system (Scheme 1): (i) Within the DNA molecule, Ca^{2+} ions preferentially interact with the phosphate group, reducing the charge repulsion between the opposite strands of the double helix [103]. (ii) Both, negatively charged lipid and protein components of AGV membranes provide binding sides for Ca^{2+} ions. Divalent metal cations are known to bind naturally to negatively charged phospholipids [104,105], neutralizing the negative charge of the phosphate group of the phospholipid headgroup. Similar to the plasma membrane [106], the interaction of Ca^{2+} ions with negatively

charged amino acids of algal membrane proteins can possibly lead to protein clustering, conformation change, or increased packing. (iii) Finally, Ca^{2+} mediated formation of aggregates of AGVs and DNA is expected. During the electrostatically driven aggregation process, a topological transformation of DNA as well as AGV membranes is assumed. Binding of AGVs on surface of DNA creates a steric barrier against intercalation of EtBr into DNA. This is manifested by a decrease of EtBr fluorescence emission intensity with increasing CaCl_2 concentration at both $\lambda_{\max}$. We observed a sudden drop in fluorescence intensity already at lowest CaCl_2 concentrations; above 10 mmol L^{-1} of CaCl_2 , the decrease in intensity was within the experimental error (Fig. 3B). As expected, the total decrease at 606 nm is more pronounced, more than double the decrease at 683 nm (Fig. 3B inset). The reference sample, pure AGVs and AGVs + DNA (without EtBr), showed at 606 nm no apparent dependence on CaCl_2 concentration. This validates that the decrease observed in presence of EtBr reflects the condensation of DNA by AGVs.

Notably, Ca^{2+} itself significantly condenses DNA by inducing a chain collapse transition. The condensate forms a compact, usually highly ordered toroidal structure [107]. We observed an abrupt decrease of I_{norm} in presence of Ca^{2+} ions, almost threefold the total decrease observed in DNA + EtBr + AGVs samples. This difference is attributed to shared capacity of Ca^{2+} ions in the presence of DNA and anionic

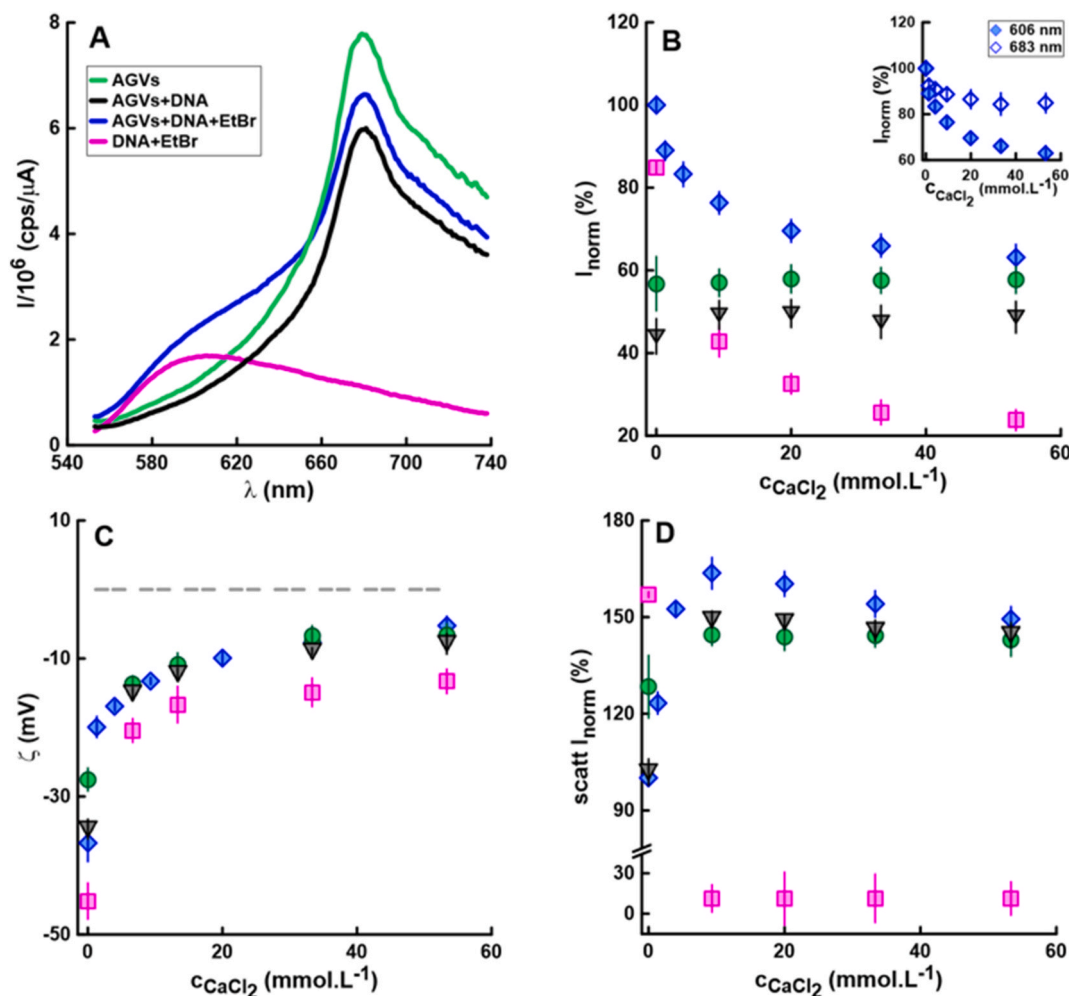


Fig. 3. (A) Emission fluorescence intensity spectra of selected samples without CaCl_2 . (B) Normalized fluorescence emission intensity I_{norm} at 606 nm, (C) zeta-potential ζ (dashed horizontal line denotes zero value), and (D) normalized scattered light intensity $\text{scatt } I_{\text{norm}}$ of the AGVs + DNA + EtBr samples as a function of CaCl_2 concentration. (B inset) Comparison of I_{norm} evaluated at 606 and 683 nm. Legend in panel A relates to all plots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

liposomes, when Ca^{2+} is able to interact either exclusively with lipids or with DNA, or simultaneously with both components [18].

It was suggested that EtBr emission intensity is directly proportional to the fraction of the DNA that is not covered with ULVs [108]. Our results indicate that at studied AGVs: DNA = 5:1 mol/base ratio about 60 % of DNA remains accessible to EtBr (Fig. 3B). This value is in agreement with results obtained for lipoplexes prepared using anionic DOPG + DOPC liposomes [18] or zwitterionic DOPC liposomes at ULVs: DNA = 1:1 mol/base [10]. Generally, loading efficiency (LE) of liposomes for highly polymerized DNA is typically low to moderate, ranging from 2 to 70 %, and is highly dependent on the size of DNA [109], loading method and formulation [110]. The relatively low binding capacity determined for AGVs in the present study can be caused by two main factors: low AGVs content in the sample, or weak Ca^{2+} – AGVs interaction, with a low binding constant.

To investigate the capacity of Ca^{2+} ions to effectively neutralise the negative charge of the studied system, zeta-potential of the AGVs + DNA + EtBr aggregates formed at different CaCl_2 concentration was measured. We found that zeta-potential nonlinearly increases from -36.7 ± 2.7 mV up to -5.3 ± 1.4 mV in the studied concentration range, with an abrupt increase up to 10 mmol L^{-1} CaCl_2 (Fig. 3C). Similar dependence was observed also for the reference systems: pure AGVs, AGVs + DNA and DNA + EtBr, but values obtained for DNA + EtBr were systematically slightly lower compared to other systems. It follows that Ca^{2+} ions are capable to moderate the negativity of the studied systems, however total elimination or, eventually, conversion of charge was not observed even at highest CaCl_2 concentrations used. As mentioned above, in the lipid components of AGVs, saturated and unsaturated acyl chains are almost equally distributed. The binding of Ca^{2+} to the lipid weakens with degree of unsaturation of the acyl chain [104, 105], as well as in the fluid phase compared to the gel phase lipid [111]. Moreover, interaction of Ca^{2+} ions with protein components of studied AGVs can also affect their overall binding constant. Algal membranes contain diverse types of channels, efflux proteins and sensors involved in Ca^{2+} -dependent signaling pathways [112]. These are made up of different kinds of amino acids, mostly glutamic acid, aspartic acid and leucine [113], which are negatively charged at physiological pH. Binding of Ca^{2+} ions is thus driven electrostatically. Biological functions of some cytosolic proteins are also dependent on Ca^{2+} binding [114]. Variation of Ca^{2+} concentration over time affects protein conformation and/or enzyme activity. Indeed, Ca^{2+} ions were found to bind to chloroplast *a* and induce its structural changes [115]. In conclusion, we assume that metal cations of higher valency could provide more effective binding of proteolipid AGVs to DNA.

The size of the aggregates was monitored nephelometrically, by measuring the intensity of the inelastic scattering on the AGVs + DNA + EtBr + CaCl_2 aggregates at right angle to the incident beam. This experiment provides information about relative changes in aggregate size. The intensity of scattering by pure AGVs was set as 100 %. Our results show that the size of aggregates rises by more than 50 % with the increase of CaCl_2 concentration to 10 mmol L^{-1} (Fig. 3D), what suggests a progressive packing of DNA by AGVs. Contrary to this, above 10 mmol L^{-1} a slight decrease in relative size of aggregates is observed. At the same time, for pure AGVs a slight increase, while for pure DNA a dramatic drop to ~ 10 % was observed already at the lowest CaCl_2

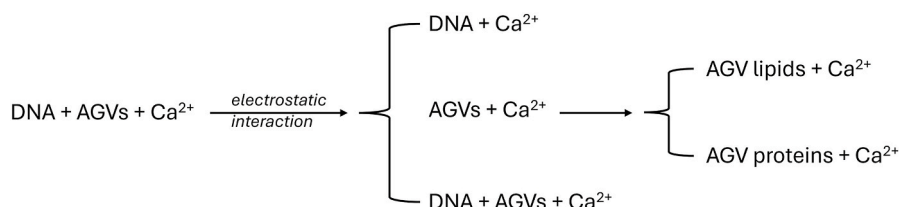
concentration examined, with no further change upon CaCl_2 concentration (Fig. 3D). These results indicate that above 10 mmol L^{-1} of CaCl_2 condensation of aggregates by Ca^{2+} ions occurs without further binding of AGVs, making the supramolecular AGVs + DNA aggregates more compact. This, together with a continuous moderate increase of zeta-potential above 10 mmol L^{-1} of CaCl_2 (Fig. 3C), indicates that the low binding capacity determined for AGVs might be alternatively caused by underestimated amount of AGVs used in our study. The bound fraction of DNA could thus be enhanced also by increasing the AGVs:DNA ratio, as suggested previously for synthetic lipid:DNA systems in presence of different Met^{2+} [16].

3.5. Interaction of reconstructed AGVs with BSA

It was suggested that solvent-free procedures should be the primary approach to prepare protein-based formulations [26]. Therefore, the loading of AGVs by direct mixing with the BSA suspension in water medium was applied, and its leakage was monitored. The two tryptophan residues (Trp134 and Trp213) in BSA have autofluorescence with emission maxima at 348 nm in pure water [116]. The fluorescence of tyrosines is very weak compared to that of tryptophanes [117]. A calibration curve based on the fluorescence intensity maximum at 350 nm, away from the autofluorescence of the AGVs (Fig. 3A), served to determine the concentration of BSA during the loading and leakage experiments (Fig. S5). Both the stability of the starting BSA solution (0.04 mM) and the first unabsorbed/unadsorbed fraction from the supernatant were checked by measuring their fluorescence intensity in real time (Fig. 4 inset). In both cases, the signal did not decay and the alteration of the fluorescence intensity of the control sample was within ± 0.4 %, so the degradation factor of BSA is negligible, and the whole leaking process (new supernatant in every period) could be followed on the same sample.

We found that 60 % of the BSA remained in the supernatant, while 40 % was in the AGV pellet (bound to AGVs). We have calculated that 3.1×10^9 BSA molecules were physically or chemically bound by AGVs (at 3.733 mg AGVs and $4 \times 10^{-9} \text{ mol}$ BSA per sample). In the leakage experiments, water was added to the “loaded” AGVs pellet, after removal of the supernatant and the after incubation period the concentration of the (newly released) BSA in the supernatant was determined. After this step, the measured supernatant was retransferred to the AGV pellet and then the incubation period and measurement were repeated. This procedure was continued until the concentration of the released BSA reached saturation (Fig. 4). This happened after the 8th cycle at a BSA concentration of $0.7 \mu\text{mol L}^{-1}$ in the supernatant. This amount of BSA (4.5 %) was probably weakly adsorbed on the surface of AGVs by secondary binding forces. Most of the BSA (95.5 %) could not be removed, neither under these conditions, nor with additional sonication during the incubation period.

BSA molecules can be chemically bound to the surface of AGVs or absorbed/adsorbed to AGV membrane bilayer. BSA is moderately hydrophobic, having a hydrophobic interior and polar patches on its surface. Its overall hydrophobicity is influenced by factors like pH, temperature, and the presence of ions [118,119], which can cause it to unfold and expose its hydrophobic residues. Thus, hydrophobic interactions of BSA and the AGV proteolipid membrane are assumed to



Scheme 1. Binding events assumed during the DNA + AGVs + Ca^{2+} aggregate formation, based on accessible electrostatic interactions.

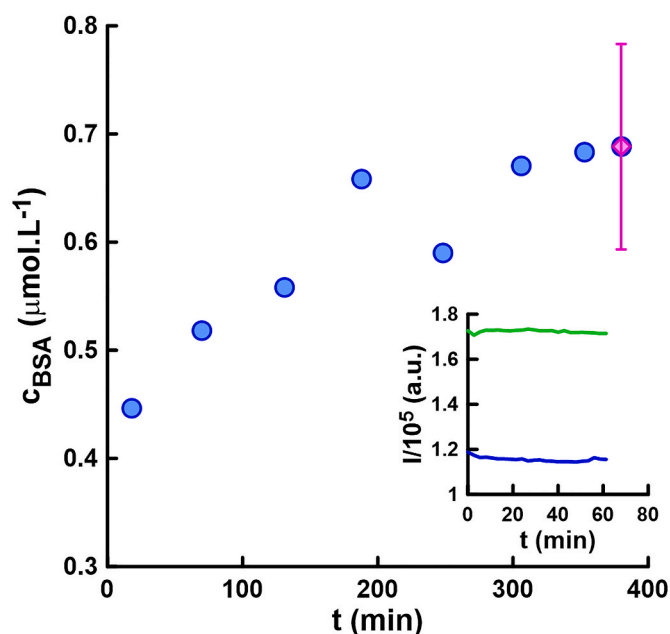


Fig. 4. Alteration in the concentration of BSA during the leakage induced by stepwise dilution and vortexing (blue dots), and consequent sonication (pink diamond) of loaded AGVs. The error bar shows the temporal variation of the control sample for comparison. (**Inset**) Temporal evolution of fluorescence intensity monitored for control sample (0.04 mmol L^{-1} BSA; green line) and the first supernatant of AGV dispersion after loading with 0.04 mmol L^{-1} BSA (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

play a key role (Scheme 2): (i) Hydrophobicity may drive interaction of BSA with AGV membrane proteins [120] and/or (ii) BSA-BSA interactions within the membrane bilayer. However, it appears that hydration forces dominate the BSA pair interaction (Moon et al., 2000). (iii) According to the literature, hydrophobic interactions between BSA and the hydrocarbon region of the bilayer membrane are supposed to be the most significant [34]. These interactions are mediated by specific binding sites of BSA to palmitic acid and oleic acid (domain III and hydrophobic hole, respectively), as suggested within the three-domain model of the BSA structure [121]. However, the effectivity of BSA binding into liposomes is governed by a plethora of other factors, such as the lipid packing in the membrane bilayer, its surface charge, and exact chemical composition. For a brief overview, DSPC + cholesterol + dioleoyl-3-trimethylammonium propane (DOTAP) liposomes showed a maximal loading efficiency (LE) of 7.2 % after active encapsulation [122]; using soybean phosphatidylcholine liposomes containing ≥ 73 % of polar lipids a LE of 34 % was reached [123]; with liposomes prepared from a mixture of dioleoylphosphatidylethanolamine (DOPE) + cholesterol + DOTAP + polyethylene glycol LE up to 43 % was achieved depending on the hydration medium [124]. Although DOTAP-containing liposomes adsorb BSA more robustly, they lack

physical stability and tend to aggregate.

To sum up, liposome loading efficiencies for high molecular weight peptides generally range from 2 to 50 % [125]. Even advanced techniques, including freeze-thaw cycles, have not exceeded the 50 % of LE [126]. It follows, that LE of 40 % obtained in the present study for reconstructed AGVs using passive incubation method can be assumed as a moderate efficiency. To achieve higher values of LE, optimization of BSA – AGVs incubation conditions (incubation time and temperature) or application of other drug loading method, such as electroporation, freeze-thaw cycles or a use of permeabilizing agent, might be an option. Even more likely, as we assume hydrophobic interactions of BSA with the lipid components of AGV membranes, varying the protein:lipid composition in favor of the lipids could also increase the LE of reconstructed AGVs.

4. Conclusion

The main goal of the present study was to investigate the potential of algae-derived vesicles (AGVs) for the delivery of therapeutic biomacromolecules. Proteolipid vesicles have been successfully prepared from a lyophilisate of *Dunaliella tertiolecta* ghosts for the first time. Hydrated lyophilisate was treated either by extrusion or by a tip sonication to form vesicles. Extrusion of rehydrated lyophilisate suspension through a filter of defined pore size was revealed to be the most suitable method for the preparation of AGVs, due to low loss of the lipid and protein content. The reconstructed AGVs were shown to be spherical, unilamellar, with moderate polydispersity, the thickness of the membrane of $\sim 15 \text{ nm}$, and a negative surface charge of -27 mV . Thanks to chemical complexity, they retain the membrane dynamics and are likely to retain the antioxidant activity of the ‘mother’ algal cells, what might be beneficial to maintaining the chemical stability of drugs potentially transported by AGVs. The drug binding efficiency of the reconstructed AGVs upon passive loading was tested for two model biomacromolecules, DNA and BSA. The electrostatic interaction between negatively charged AGVs and DNA polyanion was mediated by Ca^{2+} ions, resulting in an effective condensation of DNA. On the other hand, the interaction of BSA with AGVs is assumed to be predominantly hydrophobic. A partial penetration of BSA molecule into the hydrophobic core of the AGV membrane is suggested. For both macromolecules tested, a binding of about 40 % of the total drug was achieved. Possible enhancement of the binding efficiency by modulation of the physicochemical conditions of the studied systems is suggested.

CRediT authorship contribution statement

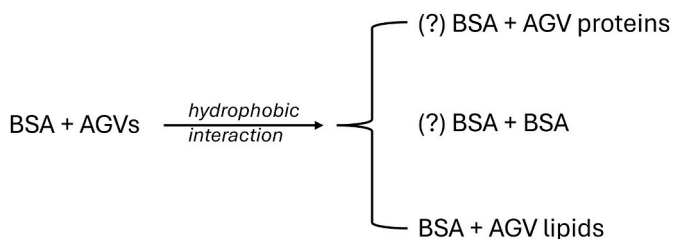
Mária Klacsová: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Krisztina Sebők-Nagy:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Tibor Páli:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization. **Marcela Chovancová:** Investigation, Formal analysis. **László Almásy:** Software, Data curation. **Daniela Uhríková:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence



Scheme 2. Binding events assumed during the BSA + AGVs aggregate formation, based on accessible hydrophobic interactions.

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jddst.2026.107985>.

Data availability

Data will be made available on request.

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