Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds†‡


Safe, efficient and specific nano-delivery systems are essential for current and emerging therapeutics, precision medicine and other biotechnology sectors. Novel bio-based nanotechnologies have recently arisen, which are based on the exploitation of extracellular vesicles (EVs). In this context, it has become essential to identify suitable organisms or cellular types to act as reliable sources of EVs and to develop their pilot- to large-scale production. The discovery of new biosources and the optimisation of related bioprocesses for the isolation and functionalisation of nano-delivery vehicles are fundamental to further develop therapeutic and biotechnological applications. Microalgae constitute sustainable sources of bio-active compounds with a range of sectorial applications including for example the formulation of health supplements, cosmetic products or food ingredients. In this study, we demonstrate that microalgae are promising producers of EVs. By analysing the nanosized extracellular nano-objects produced by eighteen microalgal species, we identified seven promising EV-producing strains belonging to distinct lineages, suggesting that the production of EVs in microalgae is an evolutionary conserved trait. Here we report the selection process and focus on one of these seven species, the glaucophyte Cyanophora paradoxa, which returned a protein yield in the small EV fraction of 1 μg of EV proteins per mg of dry weight of microalgal biomass (corresponding to 10⁶ particles per mg of dried biomass) and EVs with a diameter of 130 nm (mode), as determined by the micro bicinchoninic acid assay, nanoparticle tracking and dynamic light scattering analyses. Moreover, the extracellular nanostructures isolated from the conditioned media of microalgae species returned positive immunoblot signals for some commonly used EV-biomarkers such as Alix, Enolase, HSP70, and β-actin. Overall, this work establishes a platform for the efficient production of EVs from a sustainable bioresource and highlights the potential of microalgal EVs as novel biogenic nanovehicles.

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1. Introduction

A major therapeutic objective of modern medicine has been the development of novel treatment strategies that can target specific organs, tissues and cells.1 As such, a variety of nanoparticle-based drug delivery systems has been tested over the last decades, including synthetic polymer- and lipid-based nanoparticles as well as other organic and inorganic material-based nanovectors.2 Therapeutic agents such as RNA molecules, which are effective in vitro, often fail in vivo due to rapid clearance or biological barriers that prevent site-specific accumulation.3,4 Further, despite the appreciable success of synthetic nanomaterials to date, technical challenges involving their large-scale, cost-effective production and intrinsic toxicity still hinder their clinical and market translation.2

Biogenic nanovesicles have shown potential to naturally perform cell-specific drug release.5,6 Cell-secreted extracellular vesicles (EVs) are an example of biogenic lipid bilayer-delimited nanocarriers.7 They have been observed in many human and animal body fluids including blood, urine, saliva, semen, bronchoalveolar lavage, bile, ascitic fluid, breast milk or cerebrospinal fluid.8,9 EVs are physiological nanocarriers recognised as mediators of inter-cellular signalling by which even distant cells can exchange membrane and cytosolic contents, including proteins and RNA.7,10,11 EVs are also important mediators of cell-cell communication in conditions such as metabolic, cardiovascular, neural and neoplastic pathologies. Interestingly, EVs can act by either promoting or counteracting the disease.12,13 Moreover, they are naturally stable in various biological fluids, immunologically inert and able to pass through some biological barriers due to their small size, which could potentially overcome some of the limitations currently associated with synthetic liposomes.5,14 It has also been shown that EVs can exhibit organ-specific targeting abilities that are attributed to the interplay of several EV components.15

The finding that EVs may be used as natural carriers of small bioactive molecules has hence raised great interest from a number of scientific disciplines given that they could find promising applications for the delivery of miRNA, siRNA, mRNA, IncRNA, proteins, peptides, lipids, synthetic drugs or other cargo.16

EVs constitute vehicles for inter-species communication, as evidenced from the microbiota and human gut cell interactions, and from the interactions between plants and their pathogens; EVs have been indeed found in all the three domains of life: archaea, bacteria and eukarya.17–21 There are various cell sources available to produce EVs and indeed several have been in the process of being exploited for therapeutic applications.22 However, one attractive source that has remained largely unexplored to date is microalgae.

Microalgae are a heterogeneous group of protistean organisms of polyphyletic origins that constitute a rich reservoir of bioactive metabolites, including polysaccharides, lipids, proteins, pigments, vitamins, antioxidants and other bioactive compounds.23–25 Microalgae are perceived as renewable bioresources which have been considered for applications in a variety of fields including wastewater treatment, atmospheric CO2 sequestration, bioenergy, drug development, biofertilisation, feed manufacture or nutraceutical formulations.26–32 As such, a range of microalgae species interspersed in a variety of lineages have the capacity to synthesise high-value metabolites such as xantophyll pigments or the omega-3 long chain polyunsaturated fatty acids EPA and DHA, which have been claimed to have a wide range of beneficial health effects (e.g. antioxidant, anti-inflammation and antibacterial activities) and have high potential for niche markets.33–35 Microalgae are believed to hold a number of advantages over other photosynthetic crops as they have higher growth rates and can be cultivated on non-arable land. They also do not depend on seasonal fluctuation limitations as their growth requirements can be tailored all year round under controlled conditions in specifically designed photobioreactors.

In the context of the H2020-FETOpen project VES4US (http://www.ves4us.eu) and in the present work we propose microalgae as novel sources of EVs to be used as tailor-made products for different industrial sectors such as nutraceutics, cosmetics or nanomedicine. To this end, we developed a platform for the production, isolation and characterisation of EVs from microalgae. Our results demonstrate that EVs can be isolated from different microalgal strains, exhibiting all the key features of EVs. As an important first step towards scaling-up production, we analysed a number of species interspersed across several microalgal lineages and identified those best suited for the future biorefining of EVs.

2. Materials and methods

2.1 Microalgae strain selection and cultivation

18 microalgae strains were grown for 30 days in triplicate borosilicate glass tubes containing 60 ml of f/2 medium.36 The selection of the strains was based on including members from the main microalgal lineages as well as considering a variety of features such as seawater and freshwater inhabitants, small and large sized cells, colonial and single cells and species with sequenced genomes (ESI File 1†). The cultures were initiated with a 10% (v/v) starting inoculum from actively dividing stocks at 1.67 mg ml−1 (wet biomass). The incubation conditions consisted of a temperature of 20 °C ± 1 °C and an illumination regime of 100 μmol m−2 s−1 with a light:darkness photoperiod of 14:10. The biomass of the strains was collected at day 30 of culture by centrifugation (2000g, 10 min) and freeze-dried overnight prior to subsequent analyses. The biomass of marine species was treated with 1 ml of 0.5 M ammonium formate for desalting prior to freeze-drying.

2.2 Pigment extraction and analysis

Pigment extraction was carried out according to Mc Gee et al.37 Samples of freeze-dried biomass (2–3 mg) were mixed with 500 μl of ice cold 100% acetone and glass beads and placed in a FastPrep FP120 ribolyser for 40 s at full speed. Deionised water was added to bring the solution to 80% acetone (v/v) and
and the transfer line at 280 °C. The oven gradient temperature was as follows: an initial hold at 50 °C for 2 min followed by 20 °C min\(^{-1}\) ramp to 160 °C for 0 min, a 4 °C min\(^{-1}\) ramp to 220 °C for 5 min and finally a 4 °C min\(^{-1}\) ramp to 240 °C for 12.5 min. The mass spectrometry conditions had a solvent delay of 10.5 minutes. Identifications were carried out by comparing retention times against standards of the Supelco® 37 Component FAME Mix and using the MS NIST 08 library.

### 2.4 Isolation and characterisation of microalgal EVs

#### 2.4.1 Microalgal EV isolation.

The microalgal cultures were centrifuged on day 30 at low speed (2000g) for 10 minutes to separate cells from the culture medium. Then, the isolation of EVs from the supernatant was performed by differential ultracentrifugation (dUC).\(^{40}\) Large EV nanoparticles (lEVs) were isolated in 50 ml Eppendorf polypropylene conical tubes at 10 000g for 30 minutes at 4 °C using an Eppendorf rotor F34-6-38. The resulting supernatant was then used to isolate small EVs (sEVs) that were collected into Beckman Coulter polypropylene open top tubes via centrifugation at 118 000g for 70 minutes at 4 °C using a Beckman SW28 rotor. After a PBS washing step, the pellet was re-suspended in PBS for subsequent analyses.

#### 2.4.2 BCA assay and immunoblotting.

The protein concentration of EV nanoparticles was measured using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). This colorimetric method provides a relative concentration to a protein standard (bovine serum albumin, BSA), which is used for the preparation of a calibration curve. The relative absorbance of the BCA soluble compound was measured at 562 nm using a GloMax® Discover Microplate Reader. The signal rises linearly with protein concentration over a protein range of 20–200 μg ml\(^{-1}\).

Proteins were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg of cell lysate and EV samples (in PBS) were mixed with 5x loading buffer (0.25 M Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 0.25 M diethiothreitol (DTT) and 0.25% bromophenol blue) at 100 °C for 5 min and loaded on 10% SDS-PAGE for electrophoretic analyses. Proteins were blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with BSA-TBS-T solution (3% powdered with bovine serum albumin in TBST (50 mM Tris HCl pH 8.0, 150 mM NaCl,0.05% Tween 20)) for 1 hour at room temperature, followed by primary antibody incubation overnight at 4 °C. We tested different antibodies and found that antibodies anti-Alix (clone 3A9, dil. 1:150 in 3% BSA/TBS-T1X), anti-Enolase (clone A5, dil. 1:400 in 3% BSA/TBS-T1X), anti-β-Actin (clone AC15, dil. 1:400 in 3% BSA/TBS-T1X) and anti-HSP70 (clone W27, dil. 1:500 in 5% milk/ TBS-T1X) (Santa Cruz Biotechnology, USA), raised against different mammalian EV markers,\(^{7}\) also showed cross-reactivity to microalgae and were used in the present study. After washing, membranes were incubated for 1 hour with secondary antibodies according to the manufacturer’s instructions (horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies, cell signalling). Membranes were

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**2.3 Lipid extraction and fame analysis**

The freeze-dried microalgal biomass of the 18 strains was extracted according to Ryckebosch et al.\(^{39}\) with slight modifications. First, 400 μl of methanol was added to dried biomass (2–15 mg), followed with 200 μl of chloroform and 40 μl of deionised water. The sample was then vortexed and centrifuged (2000 rpm, 10 min). The supernatant was discarded and the bottom chloroform layer collected. The residual biomass in the tube was re-extracted using 200 μl of methanol and chloroform, vortexed and centrifuged again. The upper layer was collected and the extraction was carried out twice more on the residual biomass. The four lipid extract layers were then pooled together into a 15 ml tube and Na₂SO₄ salts added for dewatering. Upon further centrifugation, the solution was placed in a new tube and the sample was then evaporated to dryness under a nitrogen stream. The residue was then resuspended in 500 μl of chloroform : methanol (50:50) as final extract. Prior to analysis, 200 μl of sample was placed in a GC-MS vial fitted with a glass insert and supplemented with 50 μl of trimethylsulfonium hydroxide (TMSH) for transmethylation. The samples were maintained for at least 1 hour at room temperature prior to analysis by GC-MS. The separation of fatty acid methyl esters (FAMEs) in the microalgal extracts was carried out using a BPX70 120 m column with an internal diameter of 0.25 mm on an Agilent7890A/5975C GC-MS system equipped with the MassHunter software. Samples were injected at a split ratio of 100:1 at an inlet temperature of 250 °C with the helium flow rate set at 2 ml min\(^{-1}\) (48.51 psi)
washed four times in TBST for 20 min. Immunoblots were revealed using SuperSignal™ ECL (Thermo Fisher Scientific). Densitometric analyses of protein bands in the immunoblot assays were performed using the ImageJ software; biomarker optical densities (OD) were normalised against their equivalent band ODs measured in the positive control (C2C12 lysate).

2.4.3 Nanoparticle tracking analysis (NTA). Nanoparticle size distribution and concentration were measured using a NanoSight NS300 (Malvern Panalytical, UK). The instrument was equipped with a 488 nm laser, a high sensitivity sCMOS camera and a syringe pump. The EV particles were diluted in particle-free water to generate a dilution in which 20–120 particles per frame were tracked to obtain a concentration within the recommended measurement range (1–10 × 10^6 particles per ml). For each sample, 5 experiment videos of 60 seconds duration were analysed using NTA 3.4 Build 3.4.003 (camera level 15–16) with syringe pump speed 30. A total of 1500 frames were examined per sample, which were captured and analysed by applying instrument-optimised settings using a suitable detection threshold so that the observed particles are marked with a red cross and that no more than 5 blue crosses are seen. Further settings, such as blur size and Max Jump Distance were set to “automatic” and viscosity was set to water (0.841–0.844 cP).

2.4.4 Dynamic light scattering (DLS). Scattered light intensity and its time autocorrelation function g(2)(t) were measured simultaneously on different samples at 20 °C using a Brookhaven BI-9000 correlator (Brookhaven Instruments, Holtsville, NY, USA) equipped with a solid state laser tuned at 532 nm. The samples were diluted to a final total protein concentration ≤50 µg ml⁻¹ for both sEVs and lEVs to avoid vesicle interaction and multiple scattering artefacts. Absolute values for scattered intensity (Rayleigh ratio) were obtained by normalisation to toluene, whose Rayleigh ratio at 532 nm was taken as 28 × 10⁻⁶ cm⁻¹. Absolute intensity values were used to estimate the total content in small and large EVs. The intensity-averaged size distribution, namely the distribution of hydrodynamic radii D_h, was derived by fitting the autocorrelation function with a multi-peaked Schultz distribution for the particle diffusion coefficients D and then using the classical Stokes–Einstein relation \( D = (k_BT)/(3\pi\eta D_h) \), where \( k_B \) is the Boltzmann constant, \( T \) is the temperature and \( \eta \) is the solvent viscosity.\(^{41}\)

2.4.5 Scanning electron microscopy (SEM). Samples were fixed in 0.4% paraformaldehyde and 2.5% glutaraldehyde in 300 mM PBS, at 4 °C. The pre-fixed samples were applied onto polycarbonate filters with pore-diameter of 0.05 µm (STERLITECH). The EV-containing filters were post-fixed in bath following the protocol of Lešer et al.\(^ {42} \) Briefly, the primary fixatives were removed by three steps of washing with distilled water (10 min incubation in each step). Samples were then incubated for 1 hour in 2% OsO₄. They were washed with distilled water (three washing steps with 10 min incubation time), treated with saturated water solution of thiocarbohydrazide (15 min incubation time), washed again (three washing steps in distilled water, 10 min incubation time each) and subjected to 2% OsO₄ again for 1 hour. After the second incubation in OsO₄, the unbound osmium was removed in another three steps of washing (in distilled water, 10 min incubation time each). The samples were dehydrated in graded series of ethanol (30–100%, 10 min incubation in each solution; absolute ethanol was replaced three times), followed by gradual series of hexamethyldisilizane (mixed with absolute ethanol; 30%, 50% and 100%, 10 min in each solution) and finally air dried. The dried samples were Au/Pd coated (PECS Gatan 682) and examined using a JSM-6500F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan).

2.5 Decision grid for ranking strains according to their EV production potential

A decision matrix was compiled to identify the species best suited for EV production.\(^ {43,44} \) Each strain was given a score against a set number of selected criteria. A score of 0 or 1 was assigned for settling velocity based on the absence/presence of residual cells as determined by microscopy after low-speed centrifugation. The susceptibility to contamination was deemed more elevated for freshwater species (score = 1) than marine species (score = 2). Species with a sequenced genome (yes = 1, no = 0) were given extra weighting in the context of facilitating subsequent proteomic analyses. For biochemical analyses, EV protein concentration (assessed with the BCA assay) and number of particles (assessed by NTA) were given a score of 3 for protein concentration greater than 0.6 µg ml⁻¹ and number of particles per ml greater than 10^8, a score of 2 for values less than 0.6 µg ml⁻¹ and number of particles per ml between 10^7 and 10^8, a score of 1 for values equal to 0 µg ml⁻¹ and equal or lower than 10^7 particles per ml. The matrix also included information derived from biophysical analyses: DLS signal quality (scores of 0 and 1 for low and good signals, respectively) and size distribution by DLS and NTA analyses (scores of 1, 2 and 3 for wide, medium and narrow distributions, respectively). For protein markers, densitometric analyses of protein bands in the immunoblot assays were performed using the ImageJ software; biomarker optical densities (OD) were normalised against their equivalent band ODs measured in the positive control (C2C12 cell lysate). Scores of 2, 1 and 0 were assigned to each marker for OD ratios >1, <1 and = 0, respectively. Finally, strains were given scores of 3, 2 and 1 for good, medium and bad shapes and features as determined by SEM analysis (or score of 0 when not determined for samples with low EV yield and concentration).

These criteria were also given differential weights (1 = low; 2 = medium; 3 = high impact) based on their relative importance in the decision process (ESI File 2). The tallies for all the criteria were then added to give each strain a final score.

2.6 Toxicity analyses on mammalian cell lines

2.6.1 Cell cultures. C2C12 cell line is a myoblast line established from normal adult mouse muscle. MDA-MB 231 cell line is an epithelial, human breast cancer cell line. Both cell lines were obtained from ATCC (ATCC-LGC, Wesel,
Germany) and were maintained at 37 °C in a humidified atmosphere (5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) containing 15% (v/v) fetal bovine serum (FBS) (Gibco, Life Technologies) plus 2 mM l-glutamine, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Sigma-Aldrich) for the C2C12 cell line, and 10% (v/v) FBS plus 2 mM l-glutamine, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin for the MDA-MB-231 cell line.

2.6.2 Cell viability assay. Tumoral (MDA-MB 231) and normal (C2C12) cell lines were seeded in 96-well plates at a density of 2 × 10³ cells per well and maintained using suitable culture conditions. The assay was carried out with EVs isolated from Cyanophora paradoxa, which scored as one of the most promising strains in the screening analysis. Similar to other studies carried, the EVs were used at concentrations ranging 0.1 to 2.0 μg ml⁻¹. Under our experimental conditions, this is equivalent to ~10⁻⁵ to 10⁻⁶ EVs per cell, the estimated number of vesicles considered necessary to cover the surface of a cell. Thus, 24 hours after seeding, the cells were incubated for 24, 48 and 72 hours with Cyanophora paradoxa-derived EVs. The cells treated with PBS alone were used as control. Cell viability was evaluated using the CellTiter 96® AQueous one solution reagent (Promega) according to the manufacturer’s instructions. The mean optical density (OD, absorbance) of four wells in the indicated groups was used to calculate the percentage of cell viability as follows: percentage of cell viability = (Aₜreatment – A Blank)/(A Control (untreated) – A Blank) × 100 (where, A = absorbance at 490 nm). Values were expressed as means ± SD of three biological samples, each performed in triplicate.

2.6.3 Genotoxicity assay. MDA MB 2311 cells were plated onto glass coverslips and grown in DMEM complete medium for 24 hours. Cells were then incubated with 2 μg ml⁻¹ of Cyanophora paradoxa-derived EVs for 48 and 72 hours. Thereafter, the medium was removed and cells were washed twice with PBS and subsequently stained with Acridine orange/PBS solution (Sigma) at 100 μg ml⁻¹ for 10 seconds at room temperature and quickly examined by epi-fluorescence microscopy (Leica, DFC450C). Acridine orange is a cell permeating nucleic acid binding dye that emits green fluorescence when bound to double-strand DNA and red fluorescence when bound to single-strand DNA or RNA. This staining technique allows discriminating between intact (green nuclei) and damaged DNA in cells (red nuclei).

2.7 Quality management system

We developed a quality management system (QMS) compatible with UNI EN ISO 9001:2015 standard to efficiently deal with the targeted innovation level of this work, its interdisciplinarity and the multi-site structure of the study. Our QMS supported all scientific activities inside the study, including the identification and sharing of best practice and standard operating procedures (SOPs) so as to increase the reliability and reproducibility of the results as well as the overall performance of the project. Customised lab notebooks and SOP models were developed, distributed and utilised among the participating laboratories. Quality assurance and quality control activities, including checklists, audit and review meetings were regularly performed to monitor the specific activities of partners and associated deliverables and outcomes.

2.8 EV-track

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV200076).

3. Results

3.1 Pigment profiling and fame signature of microalgae

Prior to characterise the attributes of EVs isolated from the cultures, the chemical signatures of the microagal biomass of each strain were first determined in terms of pigment and FAME contents. The pigment composition of the 18 strains selected were typical of their corresponding phytoplankton groups (Fig. 1). For example, similarities in pigment profiles were visible amongst the chlorophyte species or fucoxanthin-containing chromophytes. Promising contents in the high value xantophylls fucoxanthin, lutein and zeaxanthin were identified in strains Phaeothamnion sp. LACW34, Ankistrodesmus sp. DMGFW08 and Cyanophora paradoxa CCAP981/1, respectively. The carotenoid ββ carotene was also prominent in the strain Tetraselmis chuii CCAP66/21B. Cluster analysis of the strains based on pigment profile composition largely grouped them according to their evolutionary history with two main groups, one containing the stramenopiles and a second one with two sister clades of mostly chlorophytes. An example of chromatogram obtained for the Glaucophyte species Cyanophora paradoxa is provided in Fig. 2.

The fatty acid methyl ester (FAME) signatures of the 18 microalgal strains were analysed by GC-MS (Fig. 3). The profiles were complex and showed amongst the strains substantial variation in saturated and unsaturated fatty acids content. Interestingly, the high-value long chain PUFAs EPA (C20:5) and/or DHA (C22:6) were found in 12 strains. Of those, Nannochloropsis sp. CCAP211/46, Amphidinium sp. LACW42 and Diacronema sp. GM45 showed higher contents in EPA and DHA. The clustering of the strains based on FAME composition did not group them according to their overall phylogeny but rather on their relative content in fatty acids with Ankistrodesmus sp., Kirchneriella sp., Nannochloropsis sp. and Amphidinium sp. clustering together as the species with the highest yields. An example of FAME profile obtained for the Glaucophyte species Cyanophora paradoxa is provided in Fig. 4.

3.2 Microalgal EVs: purification and characterisation

After the removal of cells and cellular debris from the microalgae-conditioned media, the samples were processed using a well-established and standardised differential ultracentrifugation (DUC) protocol. EV fractions were characterised following the recommendations of the International Society for Extracellular Vesicles known as MISEV guidelines.
yses focused on sEV enriched fractions. Specifically, dUC-isolated particles from each strain were biochemically and bio-
physically analysed in two independent experiments. As such, total protein content and the expression of selected cellular and EV markers (Alix, Enolase, HSP70, and β-actin) cross-reacting with microalgal proteins were determined together with the number and size distribution of EV particles. The morphology of the sEVs was analysed by SEM. Negative control samples with no EVs were also prepared with the same SEM protocol and did not return major artefacts (ESI File 3‡). Far more nanoobjects were visible by SEM in the microalga-
derived sEV preparations with sizes and shapes aligned with what is expected of EV morphology.

Results were used to prepare a microalgae-derived sEV identity card for each strain (ESI 4–9‡). Fig. 5 provides an example of identity card for the EV particles isolated from the glaucophyte Cyanophora paradoxa.

BCA assay-based analysis of samples from this strain returned for the sEV fraction a yield of 1.45 ± 0.3 μg of total EV-protein per ml of microalgal conditioned media, corresponding to ~2 μg of proteins per mg of dry weight of microalgal biomass. The subsequent immunoblot analyses showed strong positive signals for Alix, enolase, HSP70 and β-actin proteins in the sEV fractions. Weaker signals were observed for the lEV preparations for all EV markers. The sEV preparations showed the highest Alix expression compared to both microalgal lysates and lEVs. Particle quantification in the samples by NTA showed a high concentration of extracellular nano-objects in the conditioned medium (1.1 × 10⁹ ± 3.8 × 10⁷ sEV particles per ml in microalgal conditioned medium, corresponding to ~2 × 10⁹ particles per mg of dry weight microalgal biomass). SEM analysis of the ultracentrifuge-processed sEV samples of the conditioned medium of Cyanophora paradoxa also revealed the presence of nano-objects with expected EV morphologies. The size distribution as determined by NTA of this particular fraction showed a main population of particles with a mode of 122.0 nm and average size of 170 ± 10 nm (polydispersity index: 0.25), which corroborated the DLS results (mode: 125 nm; average size: 180 ± 10 nm; polydispersity index: 0.30).

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**Fig. 1** HPLC-UV-DAD based pigment profiling in extracts of 18 microalgae strains. The right panel delineates groups of strains based on hierarchical clustering analysis (Ward’s method and square Euclidean distance measure). Pigment presence is indicated by the ‘+’ sign. The cluster analysis was carried out after column standardisation to return values comprised between 0 and 1.

**Fig. 2** HPLC-UV-DAD chromatogram obtained for an extract of the glaucophyte Cyanophora paradoxa CCAP981/1 indicating the main pigments detected.
3.3 Identification of promising EV-producing microalgae strains

In order to select the most promising EV-producing microalgae strains, several properties related to the quality and quantity of the sEV population were considered to build a “performance” matrix for all the strains, including (i) the EV protein content, (ii) the expression of protein markers (e.g., Alix, Hsp70, enolase, β-actin), (iii) the total scattering signal or the total particle number, as measured by DLS and NTA, respectively, and (iv) the sEV average size and size range. An initial statistical analysis was carried out by computing the correlation matrix of these variables for all the strains (ESI File 10‡). The overall correlation between each pair of variables was always lower than 50%. In addition, a principal component analysis (PCA) highlighted a lack of correlation between each pair of variables measured over the observed samples with no distinct clustering of samples on the basis of the considered properties (data not shown). This confirmed the suitability of these variables as independent criteria for sample screening. As described in the methods, these EV attributes were hence considered along with other criteria to construct a matrix for the selection of the best EV-producing microalgae strains (ESI File 2‡). The sum of the weighted scores returned a final tally for each strain based on which the most promising ones could be identified (Table 1). *Cyanophora paradoxa* obtained the highest score of 31, followed by *Tetraselmis chuii*, *Amphidinium sp.* and *Rhodella violacea*. Diacronema sp., *Dunaliella tertiolecta*, and *Phaeodactylum tricornutum* also returned high scores, but below 26. *Pediastrum* sp. and *Phaeothamnion* sp. returned the lowest score of 11.

3.4 Cellular response to *Cyanophora paradoxa*-derived EVs

After identifying *Cyanophora paradoxa* as the most promising strain, we tested the cytotoxicity and genotoxicity of sEVs obtained by this species using well-established assays on a mammalian normal cell line (myoblast C2C12 cell line) and a tumoral cell line (breast cancer MDA-MB 231 cell line). Cell viability was first assessed using the MTS assay (Fig. 6). sEVs derived from *Cyanophora paradoxa* did not show toxicity both on the tumorigenic MDA-MB 231 breast cancer and C2C12 myoblast cell lines, over time and at different concentrations (Fig. 6A and B, respectively). A slightly beneficial effect of sEVs at the higher dose tested was observed, mainly after 72 hours, for the C2C12 normal cell line (Fig. 6B). This may...
result from an experimental fluctuation or may correspond to cell viability enhancement by possibly bioactive metabolites in the microalgal sEVs used, making the future characterisation of the cargo content of microalgal EVs an important task.

4. Discussion

Native and drug-loaded EVs obtained from mammalian cells (e.g., mesenchymal stem cells, MSCs) have been the focus of a rapidly growing research field known as “cell-free therapy”. As such, recent clinical trials evaluating MSC-derived EVs in different diseases (including diabetes, ischemic stroke, melanoma and lung cancer) have been ongoing, suggesting the feasibility and short-term safety of EV administration.

Alternative cell sources, including bovine milk or bacteria derived EVs, have shown limited pharmaceutical acceptability because of their provenance. In spite of the appreciable success of synthetic nanomaterials or EVs as drug delivery vehicles, technical challenges involving their large-scale, cost-effective production and intrinsic toxicity have limited to date their clinical and market translation.

In the present study, microalgae are proposed as novel and sustainable sources of EVs. Microalgae are polyphyletic unicellular organisms for which mechanisms of secretion of EVs are apparently known only in relation to primary and motile cilia/flagella; for example, in the chlorophyte *Chlamydomonas reinhardtii*, extracellular particles, named ectosomes, are derived from the flagellar membrane and are involved in flagellar resorption. However, the isolation and description of EVs obtained from photosynthetic microalgal sources do not seem to have previously been reported with much detail. The capacity for microalgae to produce EVs is somehow curious; similar to plants, microalgae possess outside their plasma membrane a cell wall of varying thickness and chemical composition, which would have been expected to act as a physical barrier to the release of EVs. Yet, both plants and microalgae such as *Chlamydomonas* sp. appear able to do so. In addition,
other major lineages of microalgae such as diatoms and dinoflagellates possess a silica frustule or a cell wall equipped with ornamental thecal plates, which might have made them less suitable as possible candidates for EV production. Yet, the present study still showed the presence of EVs in the culture medium of all the strains tested.

The nanoparticles isolated from batch cultures of microalgae were characterised using biophysical and biochemical methods, showing attributes expected of sEVs as detailed in the literature in terms of morphology, size distribution, protein content and immunoreactivity.\textsuperscript{58} sEVs from the six best-scoring strains had size distributions with modes ranging 90–160 nm (Table 1). The information and data retrieved from the screening of the selected microalgae was summarised into EV identity cards for each strain considering some of the criteria listed in the MISEV 2018 guidelines for describing EVs.\textsuperscript{7} Under the cultivation regime and conditions used in this study, the freshwater glaucophyte \textit{Cyanophora paradoxa}, marine chlorophyte \textit{Tetraselmis chuii}, marine dinoflagellate \textit{Amphidinium} sp. and rhodophyte \textit{Rhodella violacea} returned the highest scores. These results represent a milestone in microalgal EV studies and exploitation, constituting the found-

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**Fig. 6** Cytotoxicity of \textit{Cyanophora paradoxa}-derived sEVs in (A) tumoral (MDA-MD 231 cell) and (B) normal mammalian cell lines (C2C12 cells), at different concentrations and for different timing of incubation (24, 48, and 72 hours). Values were expressed as means ± SD of three independent experiments.

**Fig. 7** Epi-fluorescence microscopy images of MDA-MB 231 cells treated with 2 μg ml\textsuperscript{-1} of \textit{Cyanophora paradoxa}-derived sEVs (b and b') and untreated cells (control, a and a') for 48 (a and b) and 72 hours (a' and b'). Magnification 20×.
dations for their future production and their potential use as tailor-made bio-products.

Focusing on *Cyanophora paradoxa*, we reported here biochemical and biophysical results and carried out an evaluation of the biological activity of sEVs produced by this microalgae. Analysis of the sEV fraction isolated as a 100,000 g pellet via a dUC-based protocol returned a yield of ~2 μg of total sEV-protein (corresponding to ~2 × 10⁷ particles as per DLS and NTA measurements) per ml of microalgal conditioned media, or ~2 μg of proteins per mg of dry weight of microalgal biomass. These results are consistent with the estimate of 10⁹ EV particles per μg-protein. The subsequent immunoblot analyses showed strong positive signals for EV markers (e.g., Alix, enolase, HSP70 and β-actin). Higher IEV protein yield (3 μg proteins per ml), smaller particle number (10⁸ μg⁻¹ of IEV proteins) and substantially weaker EV marker signals were observed for the IEV preparations (10,000 g pellet of dUC protocol), suggesting the presence of contaminants in the IEV preparations. Focusing on ultracentrifuge-processed sEV samples, SEM analysis revealed the presence of nano-objects with expected EV morphologies together with other types of particles. Other methods of EV isolation such as gradient ultracentrifugation, size exclusion chromatography or tangential flow filtration may improve the purity and the homogeneity of preparations in future work. Nevertheless, the size distribution showed a main sEV population of particles with a mode of 130 ± 5 nm. These results obtained for *Cyanophora paradoxa* sEVs are in line with those obtained from plant-derived vesicles, which include both intra- and extra-cellular vesicles, as reported for citrus juice, in which EVs showed a smaller size but similar yield (2.5 μg ml⁻¹ of lemon juice). This is also aligned with the yields of 4–24 μg ml⁻¹ (or 4–16 × 10⁸ ml⁻¹) of GMP-grade MSC-derived exosomes obtained from a 250 ml bioreactor. Greater yields (451 μg ml⁻¹, or 2 × 10¹¹ ml⁻¹) were however retrieved from prolific exosome producing R16F10 tumor cells (from murine melanoma) cultured in a flask (CELLine AD1000 type, 72 ml). Nevertheless, the apparent lack of toxicity of *Cyanophora paradoxa* sEVs on mammalian cells supports the long-term possibility of using microalgae as novel bio-resources for medium- to large-scale production of EVs for human-centered applications. The development of new therapies using functionalised microalgal EVs may be conceivable once their uptake by mammalian cells is confirmed. As a further step in this direction, we developed a QMS compatible with the UNI ISO9001:2015 standard and all the experiments were performed according to the agreed standard operating procedures identified.

Microalgae constitute a rich reservoir of bioactive metabolites such as pigments and PUFAS that have already applications in a variety of sectors. The results of this study and the mechanisms underlying their ability to release EVs now allow exploiting the potential of these microalgal EVs as novel membranous bionanomaterials. Significantly, several species of microalgae have now obtained GRAS status (generally regarded as safe) and are increasingly being considered as health foods and ingredients in nutraceutical formulations.

The potential exploitation of microalgae as novel biofactories of EVs hinges on their natural and sustainable origins, making them probably more societally acceptable (and less risky in terms of bioethics) as EV sources for formulation preparations, especially when considering the medicinal and cosmetic sectors. The use of these protistean photosynthetic microorganisms as novel producers of EVs to be further functionalised as nanovehicles of bioactive chemicals has not been explored yet and appears promising. As such, biorefining pipelines could be designed on the one hand to isolate EVs from the microalgal cultivation medium and on the other hand to exploit the variety of high-value metabolites present in the residual biomass. Adapting the cultivation process from glass tubes to large reactors is not a trivial scale-up since additional growth parameters need consideration, in particular for semi-continuous production systems. However, given that microalgae can be grown in industrial contexts in thousands of litres volumes in closed photobioreactors, the refining of large amounts of microalgal EVs and their post-harvest purification will make future pre-clinical trials largely feasible.

The study of EVs is a rapidly expanding emerging field that still needs harmonisation with respect to the best practice approaches needed to isolate, purify, store and characterise such promising nano-biomaterials. Further investigations are now needed to acquire more in-depth physiological and biochemical knowledge on microalgal EVs. Their potential bioactivity also needs to be further explored using a variety of in vivo and in vitro models together with the possibility of functionalising their membranes or loading them with bioactive molecules such as siRNA.

5. Conclusion

Extracellular nanoparticles were purified from batch cultures of several microalgae species and were characterised using biochemical and biophysical methods, indicating attributes of small EVs (e.g., exosomes) as detailed in the literature from other biological sources. This is the first biophysical and biochemical description of such membranous nanovesicles from photosynthetic microalgae. These results indicate that some strains are better suited for the isolation of EVs. Follow-on experiments will further assess their potential as new generation biogenic nanocarriers of bioactive molecules.

Conflicts of interest

There are no conflicts to declare.

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